INHERITANCE AND LINKAGE OF GEMINIVIRUS RESISTANCE GENES DERIVED FROM *LYCOPERSICON CHILENSE* (DUNAL) IN TOMATO (*LYCOPERSICON ESCULENTUM* MILL.)

BY

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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TABLE OF CONTENTS

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Doctor of Philosophy

INHERITANCE AND LINKAGE OF GEMINIVIRUS RESISTANCE GENES DERIVED FROM *LYCOPERSICON CHILENSE* (DUNAL) IN TOMATO (*LYCOPERSICON ESCULENTUM* MILL.)

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The artificial inoculation procedure for tomato mottle virus (ToMoV) was studied to determine the most effective approach for avoiding plant escapes. Examination of inoculation times suggested that a seven day inoculation time was insufficient, but with rotation of trays a nine day minimum inoculation time resulted in 100% infection of susceptible controls. Extended inoculation times over 20 days caused an increase in disease symptoms in some of the ToMoV resistant lines, suggesting a partial breakdown of resistance under heavy disease

pressure. The results suggested that a 14-day inoculation was adequate.

A resistant line derived from *Lycopersicon chilense* accession LA 1932 was crossed with a susceptible inbred (Fla. 7324) to create F_1, BC_1, BC_2 and F_2 populations. Inheritance analysis of these populations infected over two seasons suggested the action of at least two additive genes with high heritability controlled ToMoV resistance. There was no significant dominance deviation and no significant epistasis was found when data from the two seasons were combined.

ToMoV and tomato yellow leaf curl virus (TYLCV) resistant lines were screened to identify random amplified polymorphic DNA (RAPD) markers within and between genotypes derived from *L. chilense* accessions LA 1932, LA 1938, LA 1969, LA 2779. Ninety-seven polymorphisms were identified using 800 arbitrary oligonucleotide primers. Of these, 46 polymorphisms were identified in breeding lines derived from two or more of the *L. chilense* accessions screened. Analysis of segregating F_2 populations identified significant associations between ToMoV resistance and the chromosome 6 morphological marker genes self-pruning (*sp*) and potato leaf (*c*). Linkage of RAPD markers in the F_2 populations revealed three linkage groups, and these were all mapped relative to the morphological markers on chromosome 6.

Atypical yellow mosaic disease responses to ToMoV infection occurred in genotypes bred for ToMoV and TYLCV resistance. Lines exhibiting these

symptoms were screened for polymorphic markers, and 9 RAPD polymorphisms were linked to this response. The RAPD markers were also linked to a SCAR marker for tomato mosaic virus (ToMV), indicating that the region responsible for the atypical disease symptoms is located near the *Tm-2* locus on chromosome 9.

CHAPTER 1
INTRODUCTION

Geminiviruses are small DNA viruses, composed of a closed, circular, single-stranded DNA (ssDNA) genome encapsidated in twinned icosahedral nucleoprotein particles (Fig.1-1) (Goodman, 1977). There are two types, those that are monopartite with a single genomic component (DNA A) or those that are bipartite with two genomic components (DNA A and DNA B). DNA A of some viruses can replicate autonomously under some circumstances, but DNA B requires the presence of the DNA A component to replicate (Elmer et al., 1988). Geminiviruses can replicate bidirectionally through a double-stranded DNA intermediate, and both DNA A and DNA B are represented equally in the genomic single-stranded DNA and in the double-stranded DNA replicative form (Ikegami et al., 1981).

The *Geminiviridae* family has been split into three genera (*Mastrevirus*, *Curtovirus* and *Begomovirus* based on host and vector type (Table 1-1) according to the standards of the International Committee on Taxonomy of Viruses (ICTV). Several geminiviruses infect tomato in the Western Hemisphere, and these are characterized in Table A-1.

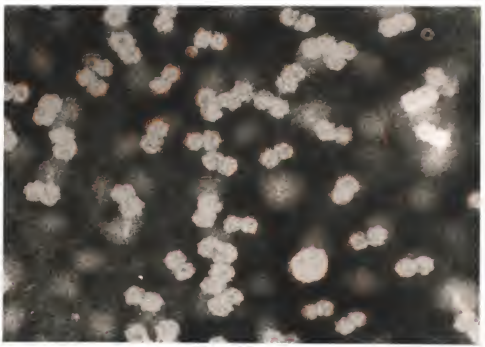


Fig. 1-1. Electron micrograph showing the twinned icosahedral structure of geminiviruses.

Table. 1-1. Classification of geminiviruses modified from Lazarowitz (1992).

Genus	Type Member	Host	Insect Vector	Genome Structure
Mastrevirus (Subgroup I)	MSV (Maize streak virus)	Monocotyledonous	Leafhoppers	Single component circular ssDNA
Curtovirus (Subgroup II)	BCTV (Beet curly top virus)	Dicotyledonous	Leafhoppers	Single component circular ssDNA
Begomovirus (Subgroup III)	BGMV (Bean golden mosaic virus)	Dicotyledonous	Whiteflies (*Bemisia tabaci, Bemisia argentifolii*)	Bipartite circular ssDNA

The geminiviruses of economic importance to the Florida tomato crop are tomato mottle virus (ToMoV)(Kring et al., 1991) and tomato yellow leaf curl virus (TYLCV)(Cohen and Harpaz, 1964). ToMoV and TYLCV are geminiviruses in the *Begomovirus* genus.

ToMoV is a bipartite geminivirus with five open reading frames (ORFs) on component A (AC1 - AC4 and AV1), and two ORFs on component B (BC1 and BV1). The ORFs of ToMoV have been mapped (Fig. 1-2). The DNA A component is composed of 2601 nucleotides that encode one ORF on the virion strand (capsid protein) and four ORFs on the complementary strand (replication proteins). The DNA B component is composed of 2541 nucleotides and encodes an ORF on the virion strand, and an ORF on the complementary strand (proteins necessary for within cell and cell to cell movement). ToMoV shows high sequence homology to Abutilon mosaic virus (AbMV), and the nucleotide sequence indicates that it is a distinct but typical geminivirus of the Western Hemisphere (Abouzid et al., 1992).

There are at least five different viruses called TYLCV, the best characterized of which is from Israel (TYLCV-I). TYLCV-I is a monopartite virus (DNA A only) and has a genome consisting of 2787 nucleotides encoding at least six ORFs: two ORFS are located on the virion strand, and four on the complementary strand (Fig. 1-3) (Navot et al., 1991).

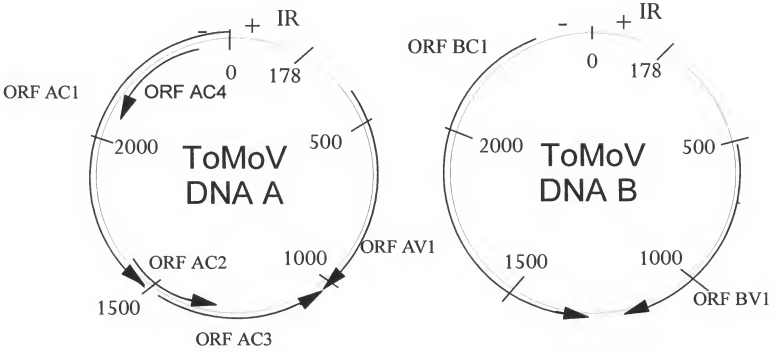


Fig. 1-2. Genomic organization of ToMoV A and B components modified from Abouzid et al., 1992. Arrows represent the length and direction of virion sense (V) and complimentary sense (C) open reading frames (ORFs). IR = intergenic region or common region.

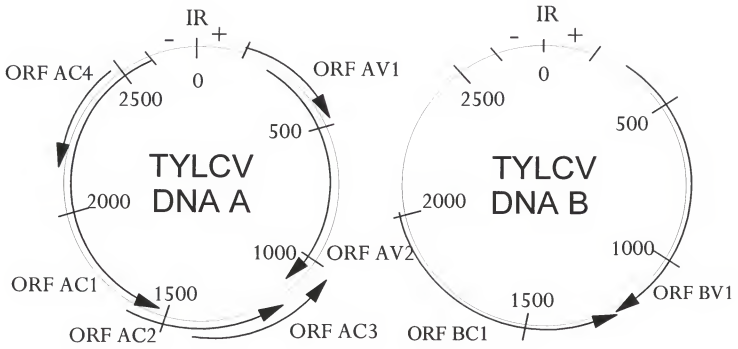


Fig. 1-3. Genomic organization of TYLCV A and B components from Pico et al., 1996. Arrows represent the length and direction of virion sense (V) and complimentary sense (C) open reading frames (ORFs). IR = intergenic region in monopartite isolates, or common region in bipartite isolates.

TYLCV is an Old World geminivirus (Stanley and Gay, 1983). The single component is weakly infectious, causing systemic infection most often through whitefly-mediated inoculation and by biolistic inoculation and agroinoculation with difficulty (Kheyr-Pour et al., 1994).

ToMoV is transmitted by *B. argentifolii.* The virus is persistent and circulative in the mature whitefly and is not acquired by immature insects. The virus is not seed transmitted (Polston et al., 1993a) and it is unlikely that the virus is transmitted through mechanical farm operations such as pruning and tying (McGovern et al., 1995). Whiteflies become viruliferous by feeding on infected plants and are able to either acquire or transmit the virus within one hour of feeding (although several hours are required between acquisition and transmission).

TYLCV is transmitted by *B. argentifolii*, and may be acquired but not transmitted by the greenhouse whitefly (*Trialeurodes vaporariorum*) and the aphid *(Myzus persicae*) (Antignus et al., 1994). TYLCV is persistent and circulative but not transmitted to the insect's progeny. Immature nymphs are able to acquire the virus and transmit it at the adult stage, and the transmission rate of females is up to six times more efficient than that of the males (Caciagli et al., 1995). TYLCV may be transmitted mechanically with low efficiency (Cohen and Nitzany, 1966), but this is unlikely to occur under field conditions. No cases of seed transmission

have been documented.

The whitefly *B. argentifolii* (a.k.a. *B. tabaci* biotype B) appeared in the Western Hemisphere in the mid-1980s and was able to reproduce and feed more efficiently on tomato than the local biotype(s) (Schuster et al., 1990). *B. argentifolii* differs from other biotypes of *B. tabaci* by gel migration distance of two enzymes (phosphoglucose isomerase and phosphoglucomutase), absence of a dorsal setae, width of thoracic tracheal folds, and width of wax extrusions from the tracheal folds at the fourth nymphal instar stage of development (Bellows et al., 1994; Perring et al., 1993). *B. argentifolii* is polyphagous with a host range of over 600 plant species and has an average adult life of 17-27 days.

The presence of golden mosaic symptoms in the leaves of weeds indigenous to Florida has been observed since the 1950s, and during the 1980s these weeds were identified as geminivirus hosts (Hiebert et al., 1995). High populations of the silverleaf whitefly *B. argentifolii* started appearing throughout Florida in the 1980s (Schuster et al., 1990). *B. argentifolii* is presumably more polyphagous than the indigenous sweetpotato whitefly biotype, feeding on over 600 plant species.

In 1989 tomato plants with unusual virus-like symptoms began appearing in southwestern Florida (Kring et al., 1989), and the pathogen was soon diagnosed as a unique whitefly-transmitted bipartite geminivirus now known as tomato mottle virus (ToMoV) (Abouzid et al., 1992). ToMoV has since been identified in

Tennessee, Virginia, South Carolina (Polston et al., 1995) and Puerto Rico (Brown et al., 1995a).

Since the appearance of ToMoV, epidemics have occurred throughout Florida, and in 1990-1991 they were estimated to have caused a 20% ($140 million) crop loss throughout the state (Schuster, 1992). Farm management practices, especially the application of foliar insecticides, helped lower the incidence of the disease. However, the introduction of the systemic insecticide imidacloprid has been most effective in controlling ToMoV epidemics (Polston et al., 1994a).

In the early 1990s, tomato crops in the Dominican Republic were devastated by another geminivirus that was confirmed as TYLCV-I (Polston et al., 1994b). Also transmitted by the silverleaf whitefly, TYLCV is a monopartite geminivirus (Kheyr-Pour et al., 1992; Navot et al., 1991; Rochester et al., 1990). First identified in Israel (Cohen and Harpaz, 1966), TYLCV is one of the most devastating diseases of cultivated tomato and has been responsible for severe production losses in the Middle East and Africa (Al-Musa, 1982). More recently, other viruses with TYLCV-like symptoms have appeared in southern Europe, threatening the tomato crops in Italy (Polizzi and Areddia, 1992) and Spain (Moriones et al., 1993).

TYLCV has been reported in other countries in the Caribbean such as Jamaica (McGlashan et al., 1994; Polston et al., 1994b), and Cuba (Ramos et al., 1996) and in July 1997 appeared in North America in southern Florida (Polston et al., 1998 submitted). The worldwide importance of TYLCV is highlighted by its geographical distribution (Table A-2; Czosnek et al., 1990).

ToMoV symptoms appear in the leaves of tomato plants about two weeks after infection. The symptoms are characterized by mottling and upward curling of the leaves. There is a reduction in leaf size, slight curling of the leaf margins combined with a paler green color and epinasty (Fig.1-4). Later the plant becomes stunted, which is caused by a reduction in the length of the internodes. The extent of stunting is greater with earlier infection. A reduction in fruit size and fruit set also occurs, the yield reduction being higher in plants infected at an early age (McGovern et al., 1995).

Fig. 1-4. Typical symptoms of ToMoV in a susceptible tomato plant.

TYLCV symptoms appear about two weeks after infection and become fully developed after a period of up to two months (Ioannou, 1985). Initial symptoms cause the tomato leaflets to curl downwards and upwards in a hook shape (Fig.1-5). The leaves become very small and appear thick and rubbery, and leaves developing later show upward curling at the leaf margins with interveinal and marginal chlorosis (Nitzany, 1975).

Fig. 1-5. Initial symptoms of TYLCV infection in a susceptible tomato plant.

The plant becomes severely stunted (the extent of stunting is greater in plants infected at an earlier age), caused by a reduction in internode length (Mazyad et al., 1979), and the top of infected plants looks like a head of broccoli (Fig.1-6). The majority of flowers abscise after infection, and infected plants produce fewer and smaller fruit.

Fig. 1-6. Typical symptoms of TYLCV infection in a susceptible tomato plant.

ToMoV has a narrow host range restricted to tomato and some members of the Solanaceae family (Polston et. al., 1993a). Although tobacco is a host, ToMoV does not currently pose a major threat to this crop. Weed hosts have also been identified, in particular tropical soda apple (*Solanum viarum* Dunal), a yellow-fruited solanaceous weed in Florida (McGovern et al., 1994; Polston et al. 1993b).

TYLCV has a significantly wider host range than ToMoV, infecting plants from six different families: *Asclepiadaceae, Compositae, Fabaceae, Malvaceae, Solanaceae* and *Umbelliferae* (Cohen and Antignus, 1994). TYLCV is primarily a threat to tomato. Understanding the host range is important in controlling the viral reservoir of ToMoV and TYLCV, particularly between seasons when tomato hosts are removed.

Several procedures are available for the diagnosis of geminiviruses which, when combined with host range and visual symptoms, can lead to an accurate identification. Reciprocal grafting of indicator plants can be used to detect the presence of the virus in infected plants. Alternatively, whitefly vectors can be used to inoculate the susceptible test plants. These procedures are time consuming and give limited precision for true diagnosis (Pilowsky and Cohen, 1974).

Epidermal strips can be analyzed under light microscopy using the nucleic acid stain Azure A (Christie and Edwardson, 1986). Azure A is used to identify DNA viruses, and the presence of characteristic inclusion bodies in the nucleus indicates infection by geminivirus. Electron microscopy provides a more precise determination of the virus family, by negative staining tissue samples with uranyl acetate and observing them under electron microscopy (E.M.) on formvar-coated copper grids (Christie et al., 1987). The unique geminate particles are very hard to identify using E.M., but their inclusion bodies can be easily distinguished from

those of other virus groups. Further diagnosis is required to distinguish between different virus species.

Serological techniques including immunodiffusion assay, enzyme-linked immunosorbent assay (ELISA) and immunospecific electron microscopy (ISEM) are generally not efficient in identifying differences between whitefly-transmitted geminiviruses and are only useful in identifying the virus genus (*Begomoviruses*). *Begomoviruses* exhibit a high degree of conservation in their capsid protein, leading to cross-reactions with monoclonal and polyclonal antibodies. Although lacking in specificity, some success has been achieved using purified preparations for serological methods (Noris et al., 1994) and a monoclonal antibody with specific affinity for BGMV as well as one with broad specificity to geminiviruses has been identified (Cancino et al., 1995).

Hybridization of specific probes can be used to identify infected plants using squash blots, dot blots or Southern blots (Gilbertson et al., 1991). Squash blots or direct-tissue blots can be used to take field samples by blotting leaves onto nylon membranes without the need for DNA extraction. They are stable and fast and can be hybridized with specific DNA probes. Dot blots may also be prepared from ground plant tissue and are more informative than squash blots as they provide a crude estimate of virus concentration and are more sensitive. Squash and dot blots are unable to identify different TYLCV isolates (Czosnek et al.,

1988); this may be achieved by digesting DNA extractions with restriction enzymes and Southern blotting on a nitrocellulose filter.

The most recent techniques for the diagnosis of geminiviruses involve the polymerase chain reaction (PCR) (Mullis et al., 1986). Geminiviruses replicate via a dsDNA intermediate, and short sequences can be amplified using degenerate oligonucleotide primers developed from conserved sequences of geminiviruses (Rojas et al., 1993). Specificity can be controlled through the primer sequence and the annealing temperature used during the PCR amplification. The procedure is powerful, as it requires only small quantities of DNA, which may be extracted from fresh, frozen or dried plant material.

Several approaches should be taken to manage ToMoV. Whitefly populations can be reduced by using good cultural practices. Tomato fields should be plowed under immediately after the final harvest to reduce whitefly populations, which multiply when pesticides are no longer sprayed, and to increase overseasoning time. It is also important to use virus-free transplants for planting, as early sources of infection can drastically reduce yields (the use of nurseries outside of growing areas helps to minimize this problem).

The chemical control of whiteflies has been difficult as they are present in several different developmental stages and whiteflies (particularly *B. argentifolii*) quickly develop resistance against insecticides (Al-Musa, 1986). Foliar pesticides

such as Thiodan, Dannitol/Monitor, Safer soap, Ambush and Asana are able to control the mature whitefly, but have little effect against the immature stages. In 1994 the systemic insecticide imidacloprid was introduced, and this has resulted in control of both ToMoV and TYLCV. Imidacloprid binds to the soil around the plant root system and and is taken up by the plant. It can be found in the youngest leaves about one week after application and becomes effective against immature whitefly after three weeks. While selection pressure for imidacloprid resistance is high, there are no current reports of resistant whitefly in Florida. However, resistance has been found in Spain (Cahill et al., 1996).

The field use of parasites or predators of whitefly is limited as the populations of natural enemies are affected by pesticides used to control other insect problems. Biological control is more effective in greenhouse situations and species that have been tested include *Encarsia formosa, Encarsia lutea* and *Eretmocerus mundus* (Gerling, 1986).

Physical barriers can be introduced such as a fine-meshed screen to prevent insect-plant contact; however, these are often associated with overheating, excessive shading and poor ventilation (Pico et al., 1996). Sometimes, it is possible to alter planting dates to avoid peak populations of the whiteflies, particularly in greenhouse situations where seasonal weather fluctuations are less important. Altering the color of plastic mulch to reflect UV light, which is

repellent to the whiteflies has also been examined (Antignus et al., 1995).

Breeding for resistant varieties is a more preferable solution than the use of chemicals. Resistant varieties may be created by introgressing resistance from wild species or more recently using pathogen-derived resistance and plant transformation.

Conventional breeding programs for geminivirus resistance have been primarily based on tolerance or resistance found in wild tomato species, as *Lycopersicon esculentum* Mill. accessions have little or no resistance to the virus. Several accessions have been identified that exhibit tolerance or resistance to TYLCV. A partially dominant tolerance gene was identified in *Lycopersicon pimpinellifolium* (L.) Mill. accession LA 121 (Pilowsky and Cohen, 1974); however, plant growth and yield are reduced in breeding lines derived from this accession compared to standard cultivars. Tolerance has since been found in other *L. pimpinellifolium* accessions (Hassan et al., 1982), and was determined to be contributed by a simple dominant gene *Tylc* (Kasrawi, 1989).

TYLCV tolerant accessions of *Lycopersicon cheesmanii* ssp. minor and *Lycopersicon hirsutum* Humb. & Bonpl. have also been identified (Hassan et al., 1984; Kasrawi et al. 1988). *L. cheesmanii* was found to contain a recessive tolerance gene, whereas *L. hirsutum* has resistance that is multigenic and dominant Hassan et al., 1984). A tolerant hybrid variety, TY-20, was released in 1988 based

on resistance derived from *Lycopersicon peruvianum* (L.) Mill. accession PI-126935 (Pilowsky et al., 1989), this tolerance delays the development of symptoms, and it was suggested that the tolerance is recessive and based on five genes (Pilowsky and Cohen, 1990).

More recently, geminivirus resistance has been identified in *Lycopersicon chilense* Dunal. (Fig. 1-7) (Zakay et al., 1991). A partially dominant resistance gene to TYLCV (*Ty-1*) was identified on chromosome 6 of *L. chilense* accession LA 1969 and was backcrossed with the aid of molecular markers (Zamir et al., 1994). Screening of accessions from several wild species with ToMoV (Scott and Schuster, 1991; Scott et al., 1995) revealed several resistant accessions and suggested that *L. chilense* was the best source for ToMoV resistance. ToMoV resistance was found in many *L. chilense* accessions, and twelve (LA 1932, LA 1938, LA 1959, LA 1960, LA 1961, LA 1963, LA 1968, LA 1969, LA 2747, LA 2762, LA 2774 and LA 2779) were crossed with tomato. Of these the most promising resistant lines were derived from LA 1932, LA 1938, LA 1961, LA 1968 and LA 2779 (Scott et al., 1995).

Fig.1-7. Morphological appearance of a wild accession on *Lycopersicon chilense.*

The association of morphological markers and their application to breeding was first suggested by Sax in 1923. Selection for phenotypic traits linked to desirable genotypes can be made, but frequently involves epistasis and linkage to undesirable traits. Progress in molecular biology has since provided several approaches to marker-assisted selection using molecular markers.

Isozymes (Market and Miller, 1959) were first used to select for several genotypes by comparing mobilities of enzymes on a starch gel. These have been gradually replaced by restriction fragment length polymorphism (RFLP) markers (Botstein et al., 1980), random amplified polymorphic DNA (RAPD) markers (Williams et al., 1990), microsatellites (Morgante and Oliveri, 1993), sequence characterized amplified region (SCAR) markers (Paran and Michelmore, 1993) and amplified fragment length polymorphism (AFLP) markers (Vos et al., 1995; Zabeau, 1993).

The new generation of markers allows more accurate and efficient linkage of molecular regions to desirable genotypes, and these markers have different characteristics that give a variety of benefits or problems.

RFLPs allow the selection of codominant polymorphisms linked to desirable genotypes, using fragments of DNA from previously mapped regions of the genome (Tanksley et al., 1992). RFLP markers are informative and reliable, even for quantitative trait loci mapping, but they are also expensive, require expertise for use of radioactivity or chemilluminescence and are not practical for screening large populations.

RAPDs are PCR-based markers created by amplifying random genomic regions with short oligonucleotide primers. RAPD markers are inexpensive, require little expertise and can be used to screen large populations, but they are

often unreliable, generally lack codominance, and their genomic location is not immediately known.

Microsatellites are simple sequence repeats highly abundant throughout the genome and can be used as probes to identify differences between closely related varieties. Microsatellites are somatically stable, codominant and widely distributed (including locations in introns, heterochromatin and noncoding DNA). They are too variable for use as molecular markers in most cases unless the lines being compared are almost identical.

SCARs are constructed from RAPD markers by cloning the amplified sequence and constructing longer more specific primers from the sequence information. SCAR markers are inexpensive, more reliable than RAPD markers and can be used to screen large populations. They are sometimes codominant and need to be linked to previously mapped markers to determine their genomic location.

AFLPs combine RFLP and PCR technology to amplify fragments from PCR adaptors ligated to the ends of restriction fragments. Very large numbers of markers can be generated using this technique, but the markers are dominant and require polyacrylamide gels and radioactivity or chemilluminescence for visualization. SCAR markers are more difficult to create from AFLPs, due to the low number of bases used to select polymorphisms (inverse PCR may be necessary

to identify flanking regions). AFLPs are most efficient for identifying differences between closely related lines, where few polymorphisms are identified using other techniques.

Molecular markers can be used to select the genotype rather than the phenotype and can be used to select plants at the seedling stage. With this technology the use of pathogens in breeding programs can be minimized, breeding can be accelerated, and plant numbers in the field can be reduced by only selecting seedlings with desirable genotypes. The use of molecular markers can improve conventional breeding programs considerably and should be an important component of a breeding program introgressing resistance genes from wild species.

Advances in biotechnology and plant transformation have provided new approaches to producing resistant varieties. The most common approach has been based on pathogen-derived resistance (PDR) (Sanford and Johnson, 1985), which uses the pathogen's own genes to create resistance in the host. Some tolerance to TYLCV has been reported when tomato is transformed with the TYLCV coat protein gene (Kunik et al., 1994), and interest has arisen regarding the control of geminiviruses with replication associated proteins (Day et al., 1991). These proteins interfere with normal viral replication even in a truncated or defective form (Anderson et al., 1992). Unlike RNA viruses, geminiviruses are within the

nucleus and have a much higher chance of becoming associated with RNA.

Of major interest has been the movement protein genes. Constitutive expression of these genes in noninfected transgenic plants resulted in disease-like symptoms, suggesting that expression of these movement protein genes is the main cause of the visual disease symptoms in the infected plant (Pascal et al., 1993; Stanley et al., 1990). Resistance to ToMoV has been observed in tobacco by transforming it with the a mutated BC1movement protein (Duan et al., 1997). Studies have shown that systemic infectivity has been reduced for African cassava mosaic geminivirus (ACMV) in tobacco transgenic for the tomato golden mosaic virus movement protein (von Arnim and Stanley, 1992).

A further understanding of host-microbe interactions needs to be developed to enhance the usefulness of this approach. Heteroencapsidation in transgenic plants has already been reported (Lecoq et al., 1993; Candelier-Harvey and Hull, 1993), suggesting that strict regulations should be maintained to avoid subsequent problems.

Slowing of disease development using PDR could prove effective alone or in conjunction with pesticides or conventional resistance; however, more regulatory hurdles exist for this technology and must also be dealt with before variety release. Other advances in biotechnology, including identification of the host resistance genes are likely to advance genetic engineering for resistance;

however, until a reliable biotechnological approach can be implemented, conventional breeding appears to be the most economic solution.

CHAPTER 2
ARTIFICIAL INOCULATION OF TOMATO MOTTLE VIRUS USING VIRULIFEROUS WHITEFLY

Introduction

Bemisia argentifolii Bellows and Perring, n. sp., appeared in the Western Hemisphere in the mid-1980s and was able to reproduce and feed more efficiently on tomato (*Lycopersicon esculentum* Mill.) in Florida than the local biotype(s) (Schuster et al., 1990). *B. argentifolii* differs from *B. tabaci* by allozyme migration distances of the enzymes phosphoglucose isomerase and phosphoglucomutase using the isoelectic focusing technique (Perring et al., 1993). Morphological differences have also been observed using scanning electron microscopy. The width of thoracic tracheal folds are constant and distinct between the two species at the forth nymphal instar stage of development, and anterior submarginal setae are present in syntypes of *B. tabaci* and almost always absent in *B. argentifolii* (Bellows et al., 1994). *B. argentifollii* induces phytotoxic responses in squash (Bedford et al., 1994); it is polyphagous with a host range of over 600 plant species and has an average life cycle of 17-27 days.

Tomato mottle virus (ToMoV) is a whitefly-transmitted geminivirus that causes a disease of tomato that is transmitted by *B. argentifolii*. The virus is

persistent and circulative in the mature whitefly, but immature insects are unable to acquire the disease. The virus is not spread through seed or through farm operations such as pruning and tying (McGovern et al, 1995). Whiteflies become viruliferous by feeding on infected plants and are able to acquire the virus within one hour, although several hours are required before the virus can be transmitted.

Another geminivirus, tomato yellow leaf curl virus (TYLCV) is transmitted by *B. argentifolii*. TYLCV is persistent and circulative in *Bemisia* spp. but is not transmitted to the progeny. Immature nymphs are able to acquire the virus and transmit it at the adult stage, and the transmission rate of females is up to six times more efficient than that of the males (Caciagli et al., 1995). TYLCV can be transmitted mechanically with difficulty and at low efficiency; but this does not occur under field conditions, and no cases of seed transmission have been documented (Cohen and Nitzany, 1966).

To accurately identify resistance in a breeding program, it is important to have a reliable inoculation procedure since natural infection may be uneven. Artificial inoculation for tomato geminiviruses may be achieved by several approaches. Mechanical inoculation (Purciful, personal communication) can be achieved for ToMoV but not TYLCV using homogenized leaf tissue in a chilled neutral pH buffer; agroinoculation can deliver a cloned copy of the viral genome in the Ti plasmid of *Agrobacterium tumefaciens* (Kheyr-Pour et al., 1994);

biolistic inoculation allows the viral DNA to be delivered to cells using high velocity microprojectiles (Klein et al., 1987); and plants can be inoculated using viruliferous *Bemisia* spp. (Pico et al., 1998).

Vector inoculation is the most frequently used approach in breeding programs because it is reliable and effective when dealing with large numbers of plants. Current approaches vary in the number of whitefly used in the inoculation and the inoculation time (Zakay et al., 1991; Pico et al., 1998; Vidavsky et al., 1998). However, evidence of actual whitefly number and distribution has not been clearly documented. It has also been suggested that mass inoculation in screened chambers is less effective than cage inoculation, which uses much smaller numbers, because whitefly preference may prevent the infection of small-leaved lines when mass inoculated (Pico et al., 1998). Pico et al. (1998) also suggested that inoculation in screened chambers overcame tolerance in lines, which could result in discarding lines with effective resistance under normal field pressure. A better understanding of the vector inoculation procedure is necessary to ensure accurate rating of plants for ToMoV resistance.

The objective of this work was to determine the ToMoV vector inoculation procedure that best differentiates resistant from susceptible tomato lines in order to accurately discern genotypes for genetic and marker studies. In doing so, the number of susceptible escapes can be determined as well as whether the resistant

lines are able to maintain high levels of resistance under heavy inoculation pressure.

Materials and Methods

A colony of viruliferous whitefly (*B. argentifolii*) was maintained on the dwarf tomato 'Florida Lanai' in a controlled temperature room at 25^{0}C. Six weeks prior to the inoculation experiments, viruliferous whitefly numbers were increased by adding five additional uninfected plants with 7-10 leaves to screened cages. Each plant was fertilized weekly with 118ml of a solution containing 4 gm of 20-20-20 N-P_2O_5-K_2O soluble fertilizer per liter of water.

ToMoV susceptible and resistant breeding lines were sown in wood flats containing Black Beauty spent coal (Real Minerals Div., Highland, Ind.) and transplanted at the cotyledon stage to styrofoam trays (3.8 cm^3 cells) prior to inoculation. The trays were moved to a screened chamber for inoculation at the 2-3 true leaf stage of development and inoculated with adult whiteflies from the infested 'Florida Lanai' plants.

Two ToMoV resistant breeding lines derived from *Lycopersicon chilense* Dunal. were used as resistant lines. Line 960744-SBK (744) was derived from accession LA 1932, and 964477-BK (477) was derived from accession LA 1938 (Table B-3). A Florida heat-tolerant breeding line Fla. 7324 (7324) was used as the susceptible control.

Inoculation experiments were performed over three seasons (Spring 1997, Fall 1997 and Spring 1998). One 'Florida Lanai' source plant was used for every four trays (512 seedlings) in Fall 1997 and Spring 1998, while one source plant was used for every two trays (256 seedlings) in Spring 1997. Whitefly counts were made to estimate the number of vectors per plant in the inoculation chamber and to gain a better idea of distribution and number during the course of the inoculation period.

Whiteflies are located primarily on the abaxial leaf surface, and counts were made at 8 a.m. to 10 a.m. on ten random plants per tray by first counting whiteflies on the stem and adaxial leaf surface, and then slowly turning each leaf at the tip and counting the number of whiteflies on the underside of the leaf. Trays were removed from the chamber at different times to analyze the effect of inoculation time, and in Spring 1998, source plants were removed proportionately to coincide with the removal of trays.

After removal from the inoculation chamber, plants were vacuumed with a portable vacuum to help remove mature whiteflies, and sprayed with the foliar pesticide thiodan (1.5 ml Phaser 3EC in 1000 ml water). After drying for several hours, the plant root zones were saturated with the systemic insecticide imidacloprid at 0.75ml per liter of water, applied with a watering can. The plants were rinsed after application to avoid foliar damage. The trays were placed in a

whitefly-free chamber until trays with longer inoculation times were ready to plant in the field.

Seedlings were planted in 20 cm-high, 76 cm-wide beds of EauGallie fine sand that were spaced on 152 cm centers, and plants were spaced 45 cm apart within rows. The beds were previously fumigated with 67% methyl bromide: 33% chloropicrin at 239 kg/ha and covered with black polyethylene mulch in the Spring and white polyethylene mulch in the Fall. Standard fertilization and seepage irrigation practices were used (Hochmuth et al., 1988), and pesticides were used according to standard practices to control foliar bacterial and fungal pathogens. Plants were staked and single tied to the stakes and no additional whitefly control was used.

Plants were rated at 21 day intervals at 42, 63 and 84 days after the beginning of inoculation (D.A.I.) for ToMoV severity according to visual symptoms on the leaves. The rating scale used ranged from zero (no visual symptoms) to four (completely susceptible), as illustrated in Table 2-1. The disease index (D.I.) was calculated as the mean disease rating of each line, irrespective of the disease incidence. The inoculation duration (I.D.) and other experimental parameters used are outlined for the three seasons below.

Table 2-1. Rating scale used to determine severity of ToMoV infection.

Rating	Symptoms
0	No disease symptoms
1	Slight symptoms visible only on close inspection
2	Symptoms apparent at a distance of 2/3m from the plant
3	Severe symptoms over entire plant
4	Stunted growth, and severe symptoms over entire plant

Spring 1997: Two resistant lines, 744 and 477, and a susceptible control, 7324, were sown on 20 January, 1997, and transplanted to trays on 3 February. They were inoculated with ToMoV for 7, 14, and 21 days beginning on 17 February, and an uninoculated control was also used to evaluate secondary field infection. Seedlings were grown in styrofoam trays with two cell sizes: 3.8 cm^3 and 5.1 cm^3. To estimate the number of viruliferous whiteflies in the inoculation chamber, two counts (7 and 14 D.A.I.) were made on 10 random seedlings per tray by slowly turning the leaves between 8 a.m. and 10 a.m. and counting the number of whiteflies on each of the plants. Counts were not made at 21 days as movement of the large seedlings caused excessive whitefly activity leading to an inaccurate count. The experiment was planted on 10 March in a completely randomized block design with 4 blocks, and 8 plants per plot.

Fall 1997: A resistant line (744), a susceptible control (7324), and a hybrid of the two lines 97E153F (F_1) were sown on 27 July, 1997, and seedlings were transplanted to trays on 11 August. They were inoculated with ToMoV for an I.D. of 7, 14 and 21 days beginning on 25 August, and an uninoculated control was used to evaluate secondary field infection. Three whitefly counts were made on 10 random seedlings per tray 7, 14 and 21 D.A.I. to estimate the number of vectors per plant in the inoculation chamber. Counts were made between 8 a.m.and 10 a.m. by slowly turning the leaves and counting the whiteflies on each plant. Trays were rotated every two days to help equalize whitefly distribution. The seedlings were planted on 16 September in a completely randomized block design with four blocks and 16 plants per plot.

Spring 1998: A resistant line (744) and a susceptible control (7324) were sown on 30 January, 1998, and seedlings were transplanted to trays on 14 February. They were inoculated with whitefly viruliferous for ToMoV for an I.D. of 3, 6, 9, 12, 15, 18, 21 and 24 days, beginning on 28 February, and an uninoculated control was used to evaluate secondary field infection. Whiteflies were counted between 8 a.m. and 10 a.m. on 10 plants per tray, 3, 6, 9, 12, 15 and 18 D.A.I., by slowly turning the leaves on each plant. Trays were rotated after each count to equalize whitefly distribution, and source plants were removed to coincide with tray removal during the inoculation to help maintain numbers.

Seedlings were planted in the field on 23 March in a randomized block design with four blocks and 12 plants per plot.

Data were analyzed using the "SAS for Windows" (SAS Institute, 1997) general linear model analysis. Experiments were analyzed as a split-plot design measured over time (Gomez and Gomez, 1984), with rating time (D.A.I.) as the main plot variable, and I.D. and line as sub-plot variables. Data were also analyzed for each line as a separate split-plot design with rating time (D.A.I.) as the main plot variable, and inoculation time as the sub-plot variable. Type III sums of squares comparisons were used to obtain F-values for the experimental variables, and Duncan's multiple range test was used to compare means.

Results

During the inoculation period the seedlings became etiolated and more tangled. Whitefly counts were more accurate during the earlier part of the inoculation when seedlings were smaller (2-3 leaf stage), as less whitefly activity was induced during the estimations. Mean whitefly numbers, and ranges, for the experiments were calculated for Spring 1997, Fall 1997 and Spring 1998 (Table 2-2).

Table 2-2. Mean number of whitefly adults per plant and range of whitefly adults estimated for Spring 1997, Fall 1997 and Spring 1998 experiments.

Season	No. sample times	Plants sampled	Mean no. +/- s.e.	Standard deviation	Range
Spring 1997	2	60	16.6 +/-2.1	16.5	1-95
Fall 1997	3	240	5.7 +/-0.3	4.7	0-29
Spring 1998	6	330	6.7 +/-0.2	3.8	1-22

The mean number of whiteflies in Spring 1997 was high due to the higher number of source plants in the inoculation chamber. The range of whitefly number per sampled plant was larger in Spring 1997, than in Fall 1997 and Spring 1998, which could have been due to the rotation of trays in the chamber in the latter two seasons. Mean whitefly numbers were significantly different for each sample period in Fall 1997 and increased throughout the inoculation (Table 2-3). This could have been caused by introducing source plants before most whiteflies were pupating or failure to remove source plants to coincide with tray removal. The Spring 1998 experiment showed the most consistent density of whiteflies throughout the experiment, as trays were rotated frequently, and source plants were removed to coincide with removal of trays.

Table 2-3. Mean whitefly numbers for each sampling period during the inoculation of plants in Spring 1997, Fall 1997 and Spring 1998.

Sample no.	Spring 1997	Fall 1997	Spring 1998
1	14.3a[z]	3.9c	8.5a
2	19.9a	6.8b	6.6b
3	-	8.9a	6.1b
4	-	-	6.0b
5	-	-	5.8b
6	-	-	5.7b

[z] Mean separation in columns by Duncan's multiple range test at $p \leq 0.05$

Symptoms on susceptible seedlings were visible after an I.D. of 14 days with viruliferous whitefly. Susceptible plants had a lighter green foliage, an abaxial curl of leaf margins and slight mottling of the leaves, when compared to seedlings of the resistant line. Susceptible plants became fully infected during the season showing severe stunting. Leaves of the susceptible plants showed a loss of green color, epinasty, mottling and a reduction in size. The majority of susceptible plants were infected during the inoculation procedure. Seedlings that 'escaped' infection in the inoculation chamber were infected by viruliferous whitefly under field conditions. Seedlings of the resistant lines were noticeably greener than those of the susceptible lines following inoculation, although some seedlings exhibited signs of infection. The majority of plants in the resistant lines remained disease free throughout the season, and the resistant plants exhibiting

signs of infection showed milder symptoms than plants of the susceptible lines.

Spring 1997: Large variation in the range of whitefly number per plant was observed on the seedlings in the inoculation chamber (Table 2-2). A large range was also observed between the trays (data not shown). Thus, the whitefly distribution was not uniform throughout the chamber, and seedlings experienced variable inoculation pressure. These results suggested that plants should be shaken, and trays rotated to help distribute the vector more evenly throughout the chamber. Plant escapes were determined (Table 2-4) and an escape rate of 2% and 1% was observed 42 D.A.I., for the 14 and 21 day I.D., respectively.

Table 2-4: Percentage of symptomless plants for the susceptible inbred Fla.7324 inoculated for different durations (I.D.) and rated at different intervals after inoculation began (Fall 1997).

I.D.	Rating (D.A.I.)[z]		
(days)	42	63	84
0	97	30	0
7	11	8	0
14	2	0	0
21	1	1	0

[z] Rating time in days after inoculation began.

All susceptible plants were infected 84 D.A.I. including those not inoculated in the chamber, suggesting a high secondary disease pressure in the

field. All plants from the 14 day I.D. were infected by 63 D.A.I., suggesting that the 14 I.D. was effective when large numbers of whiteflies were used.

The tray size was not used as a variable in the analysis as it did not have a significant effect on disease ratings, and no significant interaction was found between tray size and I.D. (data not shown). Split-plot analysis revealed significant differences between lines, I.D.s, and a line x I.D. interaction (Table 2-5). Data for the three rating times differed significantly ($p \leq 0.001$), with rating x line, and rating x I.D. interactions.

Table 2-5. Split-plot analysis of variance of Spring 1997 inoculation data.

Source	D.F.	Type III s.s.	F-value	Probability
Line	2	4898.36	1674.74	<0.001***
I.D.[z]	3	225.39	51.37	<0.001***
Line x I.D.	6	323.27	36.84	<0.001***
Error A	18	26.32	-	-
Rating[zz]	2	42.21	66.44	<0.001***
Rating x Line	4	97.32	76.60	<0.001***
Rating x	6	68.14	35.75	<0.001***
Error B	2070	657.47	-	-

[z] Inoculation duration.
[zz] Rating time in days after inoculation began.

Analysis of the split-plot data for each line (Table 2-6), indicated that the line x rating interaction was significant due to differences in the ratings of 7324.

The ratings of 744 and the 477 plants were not significantly different throughout the season, indicating that any one of the three ratings could be used to designate resistance levels of these lines. Lines 7324 and 744 both showed significant differences due to I.D., and 7324 had an I.D. x rating interaction. The differences caused by I.D. in 7324 and 744 were highlighted by the Duncan's multiple range comparisons (Table 2-7).

Table 2-6. Split-plot analysis of variance of Spring 1997 inoculation data for 744, 7324 and their F_1.

Line	Source	D.F.	TypeIII s.s.	F-value	Probability
7324	I.D.[z]	3	603.10	48.47	<0.001(***)
7324	Error A	9	37.33	-	-
7324	Rating[zz]	2	146.64	136.5	<0.001(***)
7324	Rating x I.D.	6	237.69	73.75	<0.001(***)
7324	Error B	720	386.75	-	-
744	I.D.	3	11.38	8.38	0.006(**)
744	Error A	9	4.08	-	-
744	Rating	2	2	0.63	0.39 (n.s.)
744	Rating x I.D.	6	0.41	0.20	0.97 (n.s.)
744	Error B	711	239.95	-	-
F_1	I.D.	3	0.50	2.03	0.18 (n.s.)
F_1	Error A	9	0.73	-	-
F_1	Rating	2	0.001	0.01	0.99 (n.s.)
F_1	Rating x I.D.	6	0.27	0.93	0.46 (n.s.)
F_1	Error B	639	30.77	-	-

[z] Inoculation duration.

[zz] Rating time in days after inoculation began.

Table 2-7. Disease Index (D.I.) 84 days after inoculation began (D.A.I.) for plants inoculated for 0, 7, 14 and 21 days in Spring 1997.

Duration of inoculation	Line		
	7324	744	477
0 days	1.76 b[z]	0.03 b	0.00 b
7 days	3.57 a	0.36 a	0.03 ab
14 days	3.88 a	0.29 a	0.02 ab
21 days	3.91 a	0.23 a	0.08 a

[z] Mean separation in columns by Duncan's multiple range test at $p \leq 0.05$.

Inoculated lines had significantly higher D.I.'s than uninoculated lines. A significantly higher D.I. was found in inoculated plants from line 744 suggesting the resistance was not complete in this line. Line 477 showed no significant difference in D.I.'s between inoculated and uninoculated plants. The significant I.D. x rating interaction for line 7324, was mostly due to escapes (Table 2-8).

Table 2-8. Disease Index (D.I.) for inoculation duration (I.D.) x rating interactions for susceptible line 7324 in Spring 1997.

I.D.	Rating (D.A.I)[z]		
(days)	42	63	84
0	0.05 c[zz]	1.8 c	3.47 c
7	3.38 b	3.52 b	3.83 b
14	3.75 a	3.90 a	3.98 a
21	3.82 a	3.92 a	3.98 a

z Rating time in days after inoculation began (D.A.I.)

[zz] Mean separation in columns by Duncan's multiple range test at $p \leq 0.05$.

A very low rate of infection was observed in the uninoculated control at 42 D.A.I., as they had only been in the field for 21 days and infections due to secondary spread had not yet appeared. The effects of I.D. and rating were illustrated by its infection of susceptible plants (Fig. 2-1).

The figure illustrates the effect of I.D.; if an 84 D.A.I. rating was being used, a 7-day I.D. was sufficient, as secondary disease pressure resulted in nearly 100% infection. With no secondary disease pressure in the field, the 42 D.A.I. rating was more indicative of the infection levels that were likely to be achieved. The D.I. of plants 42 D.A.I. suggested that the 14 day I.D. was more effective than the 7-day I.D., and that the 21-day I.D. did not improve the result of the 14-day I.D.

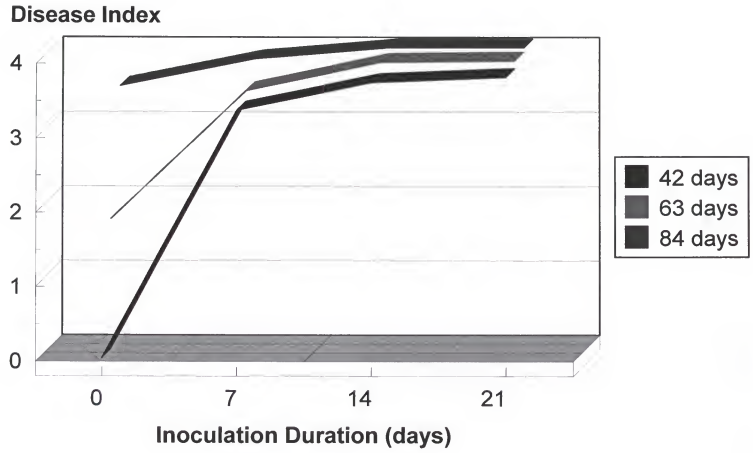


Fig 2-1. Effect of inoculation time on disease index (mean disease rating) of susceptible tomato line Fla. 7324, 42, 63 and 84 days after inoculation began (Spring 1997).

Fall 1997: A smaller variation in the range of whitefly number, and a more uniform mean count number were observed than in the Spring 1997 experiment (Table 2-2). Rotation of trays and shaking of plants created a more uniform whitefly distribution throughout the chamber. High levels of infection were observed in susceptible plants for the 49 D.A.I. rating, (83%, 98% and 100% for 7, 14 and 21-day I.D.s, respectively). A lower number of whiteflies were used (5.9) than in the Spring 1997 (16.6) inoculation. Infection was high in uninoculated plants (53 %). This high level of infection was caused by a 49 D.A.I. rating in this season (instead of a 42 D.A.I. rating) which allowed a higher level of secondary field inoculation. Whiteflies are also more active in the Fall and secondary spread is greater with increases in whitefly activity. No plants were rated worse than 2 suggesting that infection was just beginning to appear due to a high secondary disease pressure present in the field (Table 2-9).

Table 2-9. Effect of inoculation duration (I.D.) on percentage of symptomless plants for the susceptible inbred Fla.7324 rated at different times during Fall 1997.

I.D.	Rating (D.A.I.)[z]		
(days)	49[zz]	63	84
0	47	19	0
7	17	8	6
14	1	0	0
21	0	0	0

[z] Rating time in days after inoculation began.
[zz] Rated one week late.

Split-plot analysis of variance indicated significant differences between lines, I.D., and line x I.D., rating x line and rating x I.D. interactions (Table 2-10). Split-plot analysis of variance for each line indicated than I.D. significantly effected mean disease ratings of 7324, 744 and the hybrid (Table 2-11). All three lines also differed significantly during the three ratings. The rating x I.D. interaction was only significant for the resistant 744 line. The effects of I.D. (Table 2-12) and rating time (Table 2-13) were compared for each line. Lines 7324 and 744 were analyzed separately to determine the cause of the rating x I.D. interaction (Tables 2-14, 2-15).

Table 2-10. Split-plot analysis of variance of Fall 1997 inoculation data.

Source	D.F.	Type III s.s.	F-value	Probability
Line	2	2735.54	629.38	<0.001***
I.D.[z]	3	581.028	89.12	<0.001***
Line x I.D.	6	107.37	8.23	<0.001**
Error A	18	39.11	-	-
Rating[zz]	2	446.69	320.10	<0.001***
Rating x Line	4	77.92	27.92	<0.001***
Rating x I.D.	6	19.67	4.70	<0.001***
Error B	2110	1739.95	-	-

[z] Inoculation Duration.
[zz] Rating time in days after inoculation began.

Table 2-11: Split-plot analyses of variance of Fall 1997 inoculation data for lines 7324, 744 and their F_1.

Line	Source	D.F.	Type III s.s.	F-value	Probability
7324	I.D.[z]	3	293.50	30.18	<0.001***
7324	Error A	9	29.17	-	-
7324	Rating[zz]	2	210.93	165.17	<0.001***
7324	Rating x I.D.	6	6.79	1.77	0.10 (n.s.)
7324	Error B	766	1016.59	-	-
744	Time	3	59.14	21.75	<0.001***
744	Error A	9	8.16	-	-
744	Rating	2	37.44	23.92	<0.001***
744	Rating x I.D.	6	10.62	4.23	<0.001***
744	Error B	693	289.69	-	-
F_1	I.D.	3	344.77	35.76	<0.001***
F_1	Error A	9	28.93	-	-
F_1	Rating	2	290.47	140.13	<0.001***
F_1	Rating x I.D.	6	15.218	2.45	0.024*
F_1	Error B	698	723.42	-	-

[z] Inoculation Duration.
[zz] Rating time in days after inoculation began.

Table 2-12. Disease Index (D.I.) of three lines inoculated for 0, 7, 14 and 21 days in Fall 1997 (84 days after inoculation began(D.A.I.)).

Inoculation duration	Line 7324	Line 744	F_1 (7324 x 744)
0 days	2.08 c[z]	0.08 c	0.94 c
7 days	3.03 b	0.31 b	2.20 b
14 days	3.56 a	0.37 b	2.52 ab
21 days	3.63 a	0.85 a	2.65 a

[z] Mean separation in columns by Duncan's multiple range test at $p \leq 0.05$.

Table 2-13. Disease Index (D.I.) of three lines rated 49, 63 and 84 days after inoculation began in Fall 1997.

Rating (D.A.I.)[z]	Line 7324	Line 744	F_1 (7324 x 744)
49	2.34 c[zz]	0.14 b	1.23 c
63	3.31 b	0.50 a	2.31 b
84	3.56 a	0.59 a	2.72 a

[z] Rating time in days after inoculation began.
[zz] Mean separation in columns by Duncan's multiple range test at $p \leq 0.05$.

Table 2-14. Interaction of inoculation duration (I.D.) with rating time for line 7324 in Fall 1997 inoculation experiment.

	Rating (D.A.I.)[z]		
I.D. (days)	49	63	84
0	1.38 c[zz]	2.13 c	2.73 c
7	2.30 b	3.23 b	3.54 b
14	2.80 a	3.91 a	3.98 a
21	2.90 a	3.97 a	4.0 a

[z] Rating time in days after inoculation began.
[zz] Mean separation in columns by Duncan's multiple range test at $p \leq 0.05$.

Table 2-15. Interaction of inoculation duration (I.D.) with rating time for line 744.

Fall 1997	Rating (D.A.I.)[z]		
Inoculation Time	49 days	63 days	84 days
0 days	0.02 b[zz]	0.8 c	0.2 c
7 days	0.08 b	0.34 bc	0.53 b
14 days	0.14 b	0.5 b	0.51 b
21 days	0.33 a	1.08 a	1.14 a

[z] Rating time in days after inoculation began.
[zz] Mean separation in columns by Duncan's multiple range test at $p \leq 0.05$.

The results indicated that vector I.D. had a significant effect on the D.I. of all lines. There was no significant increase in the rate of infection of susceptible plants between a 14-day and 21-day I.D. However, the 14-day I.D. was significantly more effective than the 7-day I.D. (17% versus 1% escapes (Table 2-11)). The 21-day I.D. had a significant effect on 744, resulting in a higher D.I. than the 14-day I.D. (Table 2-11). This effect was highlighted in the I.D. x rating interaction for 744 (Table 2-13), which showed that the 21-day I.D. resulted in a higher D.I. at all three ratings. The higher levels of infection observed for 744 at the 21-day I.D. indicated that the disease pressure was partially breaking down the resistance in the line. Plants would be unlikely to experience this level of disease pressure under natural conditions, and it is possible that inoculating for too long a period at the seedling stage resulted in higher disease ratings for resistant plants. It is also likely that the plants were affected by higher levels of whitefly feeding at the seedling stage.

Comparison of the three ratings (Table 2-13), indicated that 7324 plants had a higher mean disease severity 63 D.A.I., and 744 plants had significantly higher ratings 49 D.A.I. These results suggested that rating earlier in the season was less effective, as plants became more infected throughout the season (Fig 2-2).

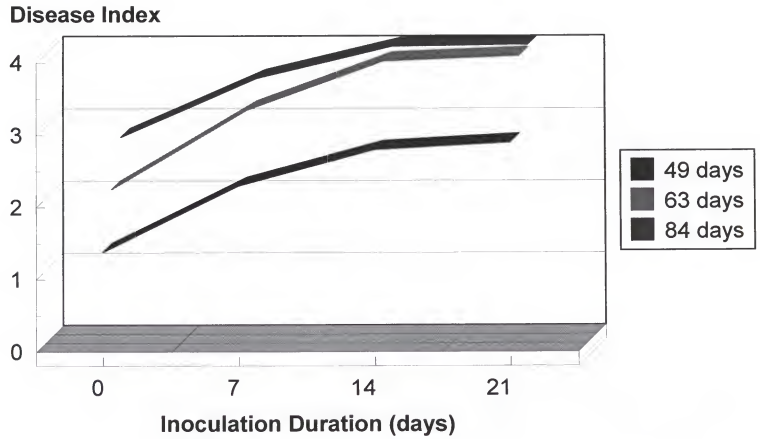


Fig 2-2. Effect of inoculation time on disease index (mean disease rating) of susceptible tomato line Fla. 7324, 49, 63 and 84 days after inoculation began (Fall 1997).

Spring 1998: Mean whitefly number was estimated (Table 2-2), and was relatively uniform throughout the course of the inoculation (Table 2-3). The more uniform distribution was due to a better distribution of source plants, rotation of the trays and shaking of the seedlings and source plants daily. A mean of 6.7 whiteflies per plant were counted throughout the course of the inoculation. Although the whitefly population was lower than that used in Spring 1997 a lower range of whiteflies per plant was observed between the seedlings (1 to 22), and an improved infection rate was observed.

The numbers of escapes were low (Table 2-16). No escapes occurred in plants with an I.D. of 9 days or longer, suggesting that steps to improve whitefly distribution had increased the percentage of infected susceptible plants, at a shorter I.D. Split-plot analysis of variance revealed significant differences between lines, I.D.s, ratings and line x I.D., line x rating and I.D. x rating interactions (Table 2-17). Split-plot analysis of variance for each line (Table 2-18) indicated that I.D. and rating time were significant variables, and a significant interaction between them occurred in 7324. Duncan's multiple range comparisons were made (Tables 2-19 and 2-20) and revealed significantly lower D.I. rates in the uninoculated controls.

Table 2-16. Effect of inoculation duration (I.D.) on percentage of symptomless plants for the susceptible inbred Fla. 7324 rated at different times during Spring 1998.

I.D.	Rating (D.A.I)[z]		
(days)	42 days	63 days	84 days
0	96	12	0
3	12	8	6
6	2	0	0
9	0	0	0
12	0	0	0
15	0	0	0
18	0	0	0
21	0	0	0
24	0	0	0

[z] Rating time in days after inoculation began.

Table 2-17. Split-plot analysis of variance of Spring 1998 inoculation data.

Source	D.F.	Type III s.s.	F Value	Probability
Line	1	5355.39	4267.34	<0.001***
I.D.[z]	8	395.09	39.35	<0.001***
Line x I.D.	8	183.75	18.30	<0.001***
Error A	24	30.12	-	-
Rating[zz]	2	340.37	376.33	<0.001***
Rating x Line	2	130.38	144.16	<0.001***
Rating x I.D.	16	61.04	8.44	<0.001***
Error B	2369	1071.33	-	-

[z] Inoculation duration.

[zz] Rating time in days after inoculation began.

Table 2-18. Split-plot analysis of variance of Spring 1998 inoculation data for lines 744 and 7324.

Line	Source	D.F.	Type III s.s.	F-value	Probability
7324	I.D.[z]	8	394.72	131.86	<0.001***
7324	Error A	24	8.98	-	-
7324	Rating[zz]	2	445.84	1159.14	<0.001***
7324	Rating x I.D.	16	102.76	33.40	<0.001***
7324	Error B	1185	227.90	-	-
744	I.D.	8	183.23	10.05	<0.001***
744	Error A	24	54.70	-	-
744	Rating	2	24.74	17.36	<0.001***
744	Rating x I.D.	16	7.80	0.68	0.81
744	Error B	1184	843.43	-	-

[z] Inoculation Duration.
[zz] Rating time in days after inoculation began

Table 2-19. Effect of inoculation duration (I.D.) on disese index (D.I.) for susceptible (7324) and resistant (744) tomato lines 84 days after inoculation (D.A.I.) began in Spring 1998.

Inoculation Time	7324	744
0 days	1.93 c[z]	0.25 cd
3 days	3.11 b	0.10 d
6 days	3.65 a	0.50 b-d
9 days	3.67 a	0.23 cd
12 days	3.67 a	0.40 cd
15 days	3.67 a	0.59 cb
18 days	3.67 a	0.49 b-d
21 days	3.67 a	0.81 b
24 days	3.67 a	1.43 a

[z] Mean separation in columns by Duncan's multiple range test at $p \leq 0.05$.

Table 2-20. Effect of rating time on disease index (D.I.) for susceptible (7324) and resistant (744) tomato lines in Spring 1998.

Rating (D.A.I.)[z]	Line 7324	Line 744
42	2.58 c[zz]	0.34 b
63	3.78 b	0.62 a
84	3.87 a	0.63 a

[z] Rating time in days after inoculation began.
[zz] Mean separation in columns by Duncan's multiple range test at $p \leq 0.05$.

Results for this season indicated that a 6-day I.D. was sufficient to achieve 98% infection of the susceptible control at 42 D.A.I., and 100% at 63 D.A.I. (Table 2-16). The shorter I.D. to obtain 100% infection than previous experiments was most likely due to improvements made in the inoculation procedure to improve the whitefly distribution. I.D.'s 21-days or longer resulted in significantly higher D.I. of the resistant line 744. This provided further evidence that over-inoculation could cause a break down in disease resistance.

The results of the three ratings again indicated that the highest D.I. in 7324 was noticeable at 63 and 84 D.A.I. (Table 2-20), whereas 63 D.A.I was a long enough time to gain accurate ratings of the resistant line (744). The I.D. x rating interaction was caused primarily by the uninoculated control (Table 2-21). The optimum I.D. and rating for gaining an accurate reading of resistance levels was highlighted in the graphs comparing rating times and I.D.'s in the susceptible controls (Figs. 2-1, 2-2, 2-3). The effect of extended inoculation time on the

resistant line 744 is also shown for Sping 1998 (Fig. 2-4). Data indicated that an I.D. of at least 7-days was necessary for an effective inoculation, but an I.D. of 14 days would be advisable.

Table 2-21. Disease Index (D.I.) resulting from combinations of inoculation duration (I.D.) with rating time for susceptible genotype Fla. 7324 in the Spring 1998 inoculation.

I.D. (days)	**Rating (D.A.I)[z]**		
	42 days	63 days	84 days
0 days	0.02 c[zz]	3.14 c	2.61 c
3 days	2.25 b	3.65 b	3.43 b
6 days	2.95 a	4.0 a	4.0 a
9 days	3.0 a	4.0 a	4.0 a
12 days	3.0 a	4.0 a	4.0 a
15 days	3.0 a	4.0 a	4.0 a
18 days	3.0 a	4.0 a	4.0 a
21 days	3.0 a	4.0 a	4.0 a
24 days	3.0 a	4.0 a	4.0 a

[z] Rating time in days after inoculation began.

[zz] Mean separation in columns by Duncan's multiple range test at $p \leq 0.05$.

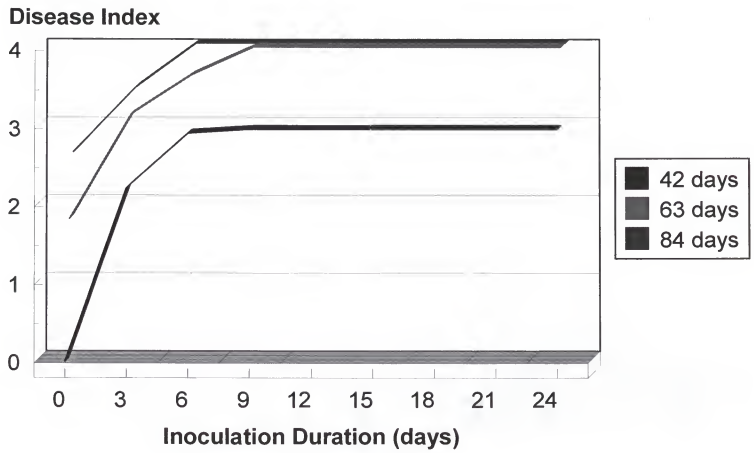


Fig 2-3. Effect of inoculation time on disease index (mean disease rating) of susceptible tomato line Fla. 7324, 42, 63 and 84 days after inoculation began (Spring 1998).

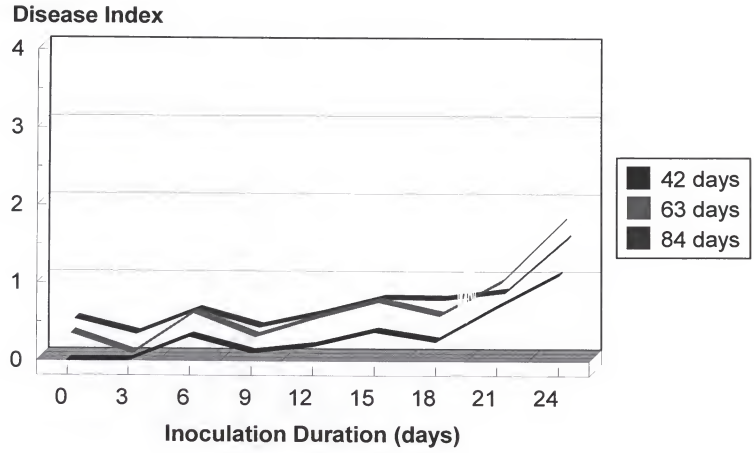


Fig 2-4. Effect of inoculation time on disease index (mean disease rating) of resistant tomato line 719 (LA 1932), 42, 63 and 84 days after inoculation began (Spring 1998).

Discussion

A reliable inoculation procedure is an important component of a geminivirus resistance breeding program, and optimizing the procedure is necessary to accurately separate resistant and susceptible plants. Inoculation using viruliferous adult whiteflies is the most efficient inoculation procedure currently available for infecting large numbers of tomato seedlings with ToMoV. To improve the efficiency of the inoculation, experimental treatments which could influence mean disease severity were explored and the experiments undertaken revealed several areas in the inoculation procedure which could lead to inconsistencies.

The dwarf tomato cultivar 'Florida Lanai' was used as source plants for infection of seedlings in the inoculation procedure. The number of whiteflies introduced on each source plant was observed to be highly variable, which would lead to inconsistencies in the number of adult whiteflies introduced to the chamber. Whiteflies continue to develop on source plants during the course of the inoculation, and the use of consistent numbers of whiteflies in different seasons or throughout the inoculation period is not practical. The whitefly distributions were inconsistent throughout the inoculation chamber, with several other possible variables including temperature, light, plant size, plant density, leaf size and color influencing the number of vectors feeding on a particular plant.

A uniform distribution of adult whiteflies is not possible without the use of clip-cages on individual plants, which is not practical for large numbers of plants. Even though the experimental error was large, estimating the number of whiteflies per plant in the chamber could help to determine if the number provided was sufficient to produce an effective inoculation.

Whitefly numbers were significantly different between samples in Fall 1997 although results from Fall 1997 and Spring 1998 suggested that a mean of 6-7 whiteflies per plant was sufficient to produce an effective inoculation, using an I.D. of at least 9-days, and preferably 14-days (Tables 2-14, 2-20) with rotation of flats and disturbance of plants.

The vector inoculation procedure at GCREC currently involves secondary field inoculation to ensure 100% infection of susceptible controls. Field inoculation can result in infection of plants in other tomato trials, and can be a potential disease reservoir for the local tomato growing districts. To determine the efficiency of the chamber inoculation, seedlings were sprayed with the foliar insecticide thiodan to kill the mature adults, and drenched with imidocloprid to kill the immature whiteflies. The rating at 42 D.A.I. provided a good representation of the percentage infection attributed to the chamber inoculation in Spring 1997 and 1998, as minimal field inoculation had occurred at this time. In Fall 1997, uninoculated plants were already beginning to show symptoms at 49

D.A.I. This was caused in part by greater activity of whiteflies due to higher temperature, suggesting that vector populations from other experiments in the field were high early in this season. No uninoculated plants were rated worse than a 2, indicating recent infection. The results showed that an I.D. of 21-days was 99.7% effective over the three seasons, an I.D. of 14-days was 99.3% effective over the three seasons, and an I.D. of 7-days was only 90% effective. These results suggest that an I.D. of 7-days is not sufficient if secondary field inoculation is not used, but a 99+% inoculation rate is achieved using only the 14-day I.D. The results also indicated that a 21-day I.D. overcame the resistance for some of the plants, which led to a significantly higher D.I. (Tables 2-17, 2-18). This occurred during Fall 1997 and Spring 1998 when plants were shaken, and trays were circulated in the inoculation chamber. The data indicated that a 21-day I.D. was too long, as it partially overcame the resistance in some of the lines leading to an inaccurate assay of genetic potential for resistance. The disease pressure endured during the 21-day I.D. was higher than the natural disease pressure to which plants are likely to be exposed to as seedlings. If a partial break down in the resistance occurs due to an unnaturally high inoculation, a potentially valuable plant may not be selected. If such plants are being evaluated for molecular markers some precision would be lost.

The results indicated that an inoculation procedure should provide a near

100% infection of susceptible plants, but not be excessive and cause symptoms in resistant plants. The graphs comparing I.D. with the susceptible line (Figs.2-1, 2-2, 2-3), indicated that an I.D. of 14-days was sufficient in all of the experiments for an effective inoculation.

It has been suggested that whiteflies selectively ignore small-leaved plants during mass inoculation in a screened chamber (Pico et al., 1998). Whitefly preference could cause inaccurate designation of resistance levels in plants, particularly in lines with wild characteristics. However, large numbers of whiteflies have been observed on small-leaved plants in the field, and wild accessions of *L. chilense* with very small leaves became infected in previous experiments (Scott and Schuster, 1991).

Several reports have indicated that ten whiteflies per plant were used for inoculation with TYLCV (Zakay et al., 1991; Zamir et al., 1994), no data were provided to support the number. The current studies indicated that large variations in the range and density occurred during inoculations and between seasons. Inoculation in 61 cm x 61 cm cages was suggested as the most reliable inoculation procedure by Pico et al. (1998); however, screening of large numbers of plants with a consistent number of whiteflies per cage using this procedure would be inefficient and labor intensive.

Several variables may have led to experimental error in the three seasons including whitefly number and distribution, environmental differences and secondary field pressure. By using large plant populations, it was possible to determine the most effective inoculation duration (14-days), and the best rating time for determining the effect of the chamber inoculation (42 D.A.I.). Practices to improve the distribution of whiteflies throughout the chamber led to a more effective inoculation (Spring 1998) and elimination of susceptible escapes by using an I.D. in excess of 9-days (Table 2-16). The distribution of whiteflies throughout the chamber was highly variable, and a mean number of 6-7 whiteflies per plant was sufficient for the I.D. suggested.

Future investigations into the vector inoculation procedure should be planted in areas free of secondary field inoculation to more accurately determine the effectiveness of the chamber inoculation, and look more closely at the interaction between the number of whiteflies introduced to the chamber with inoculation duration.

CHAPTER 3
INHERITANCE OF TOMATO MOTTLE VIRUS RESISTANCE DERIVED FROM *LYCOPERSICON CHILENSE*

Introduction

Tomato mottle geminivirus (ToMoV) first appeared in Florida in the late 1980s (Kring et al., 1989) and has been responsible for sporadic epidemics in the fresh market tomato (*Lycopersicon esculentum* Mill.) industry with yield losses of up to 20% (Schuster et al., 1992). ToMoV is a silverleaf whitefly (*Bemisia argentifolii* Bellows and Perring n. sp.) transmitted bipartite geminivirus causing leaf curling, epinasty, stunting of growth, a reduction in leaf size and a reduction in yield (Abouzid et al., 1992). Tomato yellow leaf curl virus (TYLCV) is a monopartite geminivirus with several variants prevalent throughout the Mediterranean basin (Kheyr-Pour et al., 1992; Navot et al., 1991; Rochester et al., 1990). First identified in Israel (Cohen and Harpaz, 1966), TYLCV is one of the most devastating diseases of cultivated tomato and has been responsible for severe production losses in the Middle East and Africa (Al-Musa, 1982).

TYLCV is the major viral disease of tomato worldwide and several sources of resistance have been identified. LA 1969, an accession of *Lycopersicon chilense* Dunal, was reported as the best source of resistance to TYLCV in one

study (Zakay et al., 1991) and was used to introgress a partially dominant major gene (*Ty-1*), which provided tolerance to TYLCV in tomato (Zamir et al., 1994). Other sources of TYLCV resistance include *Lycopersicon pimpinellifolium* (Jusl.) Mill. (Pilowsky and Cohen, 1974), *Lycopersicon cheesmanii* Riley and *Lycopersicon hirsutum* Humb. and Bonpl. (Kasrawi et al., 1988; Hassan et al., 1984) and *Lycopersicon peruvianum* (L.) Mill. (Pilowsky et al., 1989).

In 1990, wild *Lycopersicon* accessions were screened for resistance to ToMoV, and the best resistance was found in accessions of *L. chilense* (Scott and Schuster, 1991). Resistance to ToMoV was found in several *L. chilense* accessions, with the most promising resistant lines derived from accessions LA 1932, LA 1938, LA 1961, LA 1968 and LA 2779. Introgression of ToMoV resistance to tomato resulted in a low percentage of backcross plants in the higher resistance categories (19 of 555, or 3.4%), indicating that ToMoV resistance was controlled by more than one gene (Scott et al., 1995).

Previous studies on TYLCV resistance genes have revealed different types of genetic control including partially dominant tolerance genes derived from *L. pimpinellifolium* and *L. chilense* (Zamir et al., 1994; Pilowsky and Cohen, 1974), a simple dominance gene *Tylc* derived from *L. pimpinellifolium* (Kasrawi, 1989), a recessive tolerance gene from *L. cheesmanii* (Hassan et al., 1984), a multigenic dominant resistance from *L. hirsutum* (Hassan et al., 1984), and a recessive

tolerance based on five genes from *L. peruvianum* (Pilowsky and Cohen, 1990). The reports have been inconsistent and contradictory, with discrepancies in the inheritance of TYLCV resistance obtained in different countries of the Middle East (Kasrawi and Mansour, 1990).

Information on the inheritance of TYLCV resistance exists, but no genetic studies have been conducted on bipartite geminiviruses, including ToMoV. It is important to gain as much information about the inheritance of ToMoV resistance as possible in order to develop an appropriate breeding strategy. Understanding the inheritance of resistance to ToMoV can give a clearer idea of the amount of genetic gain expected, and the major influences on the expression of resistance. By comparing the generation means and variances from related populations, it is possible to determine estimates of heritability, effective factor number, and components of variance involved in the resistance (Mather and Jinks, 1982). This information is of use for molecular studies, as it can determine the most efficient approach for identifying and isolating polymorphisms linked to the resistance genes. The objective of this research was to determine the mode of inheritance involved in ToMoV resistance derived from *L. chilense*.

Materials and Methods

A determinate (*sp* gene) heat-tolerant Florida breeding line Fla. 7324 (P_2) was crossed reciprocally with an indeterminate (*sp*+ gene) ToMoV resistant breeding line (719 (P_1)) derived from *L. chilense* accession LA 1932 to obtain F_1 seed (Table B-1). The F_1 was backcrossed to each parent and self-pollinated to obtain BC_1, BC_2 and F_2 seed, and the seeds were sown in wood flats containing Black Beauty spent coal (Real Minerals Div., Highland Ind.) on 29 July, 1997, (Fall 1997) and 31 January, 1998 (Spring 1998). Seeds were transplanted at the cotyledon stage to styrofoam trays (3.8 cm^3 cells) on 12 August (Fall 1997) and 14 February (Spring 1998) where they were grown to the three leaf stage.

Meanwhile, a colony of viruliferous whitefly (*B. argentifolii*) was maintained on the dwarf tomato 'Florida Lanai' in a controlled temperature room at 25^{0}C. Six weeks prior to the inoculation experiments, viruliferous whitefly numbers were increased by adding five additional uninfested plants with 7-10 leaves to screened cages. Each plant was fertilized weekly with 118 ml of a solution containing 4 gm of 20-20-20 N-P_2O_5-K_2O soluble fertilizer per liter of water. The trays were moved to a screened chamber for inoculation at the 2-3 leaf stage and were inoculated for 14 days beginning on 26 August (Fall 1997) and 28 February (Spring 1998) using one source plant for every 512 seedlings.

After inoculation seedlings were planted without removal of the whiteflies on September 9th 1997 (Fall 1997) and March 14th 1998 (Spring 1998) in 20 cm-high, 76 cm-wide beds of EauGallie fine sand that were spaced on 152 cm centers. Plants were spaced 45 cm apart within rows. The beds were previously fumigated with 67% methyl bromide: 33% chloropicrin at 239 kg/ha, and covered with a black polyethylene mulch in the Spring, and a white polyethylene mulch in the Fall. Standard fertilization and seepage irrigation practices were used (Hochmuth et al., 1988), and pesticides were used to control foliar bacterial and fungal pathogens according to standard practices. Plants were staked and single tied twice during each season.

The plants were grown in a completely randomized block design with four blocks. A total of 40 plants with 10 plant plots were used for the P_1, P_2 and F_1 generations, 120 plants with 15 plants per plot and 2 plots per block for the BC_1 and BC_2 populations, and 400 plants with 25 plants per plot and 4 plots per block for the F_2 population in each season. Whitefly were transferred to the field with the plants, and ToMoV incidence was rated at 21 day intervals at 35, 56 and 77 days after inoculation began (D.A.I.) according to the rating scale in Table. 2-1. The rating scale was modified to score intermediate plants (0.5, 1.5 etc.) to obtain better distributions of disease severity ratings. The data from the final rating taken 77 D.A.I. were used for the analysis to ensure 100% inoculation of the plants, and

to record the most accurate resistance levels of the plants.

To examine possible influence of the *sp* locus, a total of 279 plants were scored for indeterminate (*sp+*) or determinate (*sp*) plant habit. Ratings at 77 D.A.I. were analyzed using "SAS for Windows" (SAS Institute, 1997) using the general linear model analysis, and frequencies were calculated.

Generation means analysis was used to obtain information on the genetic basis of *L. chilense* derived ToMoV resistance. The adequacy of the additive-dominance model was tested using the joint scaling test (Cavalli, 1952), which uses weighted least squares estimates based on the generation means. Goodness of fit to the model was tested using the chi-square distribution; and if the joint scaling test indicated adequacy of the additive-dominance model, epistatic effects were considered insignificant. The generation means analysis was based on the mean and standard error of the P_1, P_2, F_1, BC_1, BC_2 and F_2 populations according to Mather and Jinks (1982), and the analysis was calculated using a spreadsheet program (Ng, 1990). Using this program, the number of effective factors (k) was calculated (Wright, 1934), and estimates of broad-sense heritability (Mahmud and Kramer, 1951) and narrow-sense heritability (Warner, 1952) were made. Calculations of environmental variation were based on variation of the P_1, P_2 and F_1 populations (Allard, 1960). The data from the Fall and Spring seasons were compared by analysis of variance (SAS Institute, 1997) to confirm their homology

between the two seasons. The data sets were then pooled to allow a more powerful analysis using a larger population size.

Results

The susceptible breeding line Fla. 7324 (P_2) was 100% infected 77 days after inoculation began in both seasons. The resistant line 719 (P_1) showed slight infection in many of the plants, suggesting that the resistance of P_1 was not complete, or it was highly tolerant of the virus. All infected P_1 plants maintained resistance throughout the season, and no P_1 plants were rated over 2 (symptoms apparent at a distance of 2/3 m from the plant). P_1 had a larger variation than P_2 suggesting that P_1 was not completely resistant, whereas P_2 was completely susceptible. The F_2 variance in Fall 1997 was also higher than in the Spring 1998 population (Tables 3-1, 3-2).

Table 3-1. Plant number, mean, variance and standard errors for generations derived from the cross 719 (LA 1932) x Fla. 7324 in Fall 1997 (77 D.A.I.).

Generation	Number	Mean[z]	Variance	Std. error
P_1 (719)	34	0.60	0.53	0.12
BC_1	116	1.36	0.44	0.06
F_1	40	2.50	0.22	0.07
F_2	396	2.30	1.05	0.05
BC_2	119	3.34	0.39	0.06
P_2 (7324)	38	3.99	0.01	0.01

[z] Mean disease severity.

Table 3-2. Plant number, mean, variance and standard errors for generations derived from the cross 719 (LA 1932) x Fla. 7324 in Spring 1998 (77 days after inoculation began).

Generation	Number	Mean[z]	Variance	Std. error
P_1 (719)	39	0.83	0.32	0.09
BC_1	114	1.51	0.34	0.05
F_1	38	2.49	0.26	0.08
F_2	393	2.36	0.55	0.04
BC_2	119	3.00	0.42	0.06
P_2 (7324)	39	3.99	0.01	0.02

[z] Mean disease severity.

Plants were scored for *sp* and segregated indeterminate (*sp*+): determinate plants (*sp*) at a ratio of 204:75, which fitted an expected ratio of 3:1 ($\chi^2 = 0.50$ [d.f.1], $p>0.1$). The mean disease severity of determinate plants (3.04) was significantly higher ($p<0.001$) than the mean disease severity of indeterminate plants (2.04) (data not shown). The disease severity frequencies were calculated (Fig. 3-1).

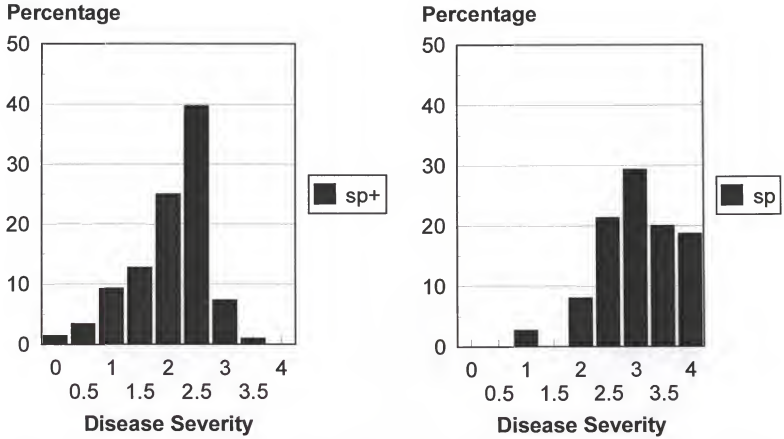


Fig. 3-1. Frequency distributions ofmean disease severity for indeterminate (sp+) and determinate (sp) plants for F_2 populations from the cross 719 (derived from accessionLA 1932) x 7324 (Fall 1997 and Spring 1998 combined).

No *sp* plants were identified in the highest resistance category by 77 D.A.I.; however,10.7% of *sp* plants rated two or better indicating that *sp* plants segregated for ToMoV resistance. The results suggested that breeding ToMoV resistance into determinate plants could be more challenging than breeding ToMoV resistance into indeterminate plants. The results also suggested the possibility that a ToMoV resistance gene or genes could be linked to the *sp* locus and that it might be necessary to obtain chromosomal crossovers to reduce linkage drag associated with this locus on chromosome 6.

In Fall 1997 the means of the F_1 and F_2 populations were skewed slightly to the susceptible side of the mid-parent value, and in Spring 1998 the F_1 and F_2 populations fell slightly to either side of the mid-parent value. The mean BC_1 ratings fell between the mean ratings of the P_1 population mean and the F_1 mean, and the mean BC_2 ratings fell between the mean ratings of the P_2 population mean and the F_1 mean (Figs. 3-2, 3-3). The F_2 populations segregated in a continuous pattern in both seasons with only 6.6% and 3.3% in the highest resistance categories (0 and 0.5), and 12.8% and 3.6% in the completely susceptible plants (3.5, 4) in Fall 1997 and Spring 1998, respectively. These results indicated control of ToMoV resistance was multigenic.

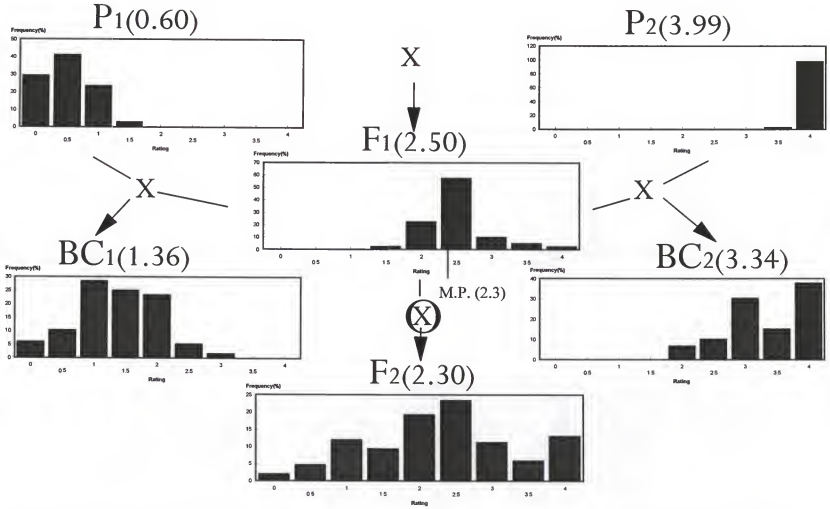


Fig. 3-2. Frequency distributions of ToMoV disease severity ratings 77 days after planting for P_1, P_2, F_1, BC_1, BC_2, F_2 generations for the cross 719 (derived from accession LA 1932) x 7324 (Fall 1997).

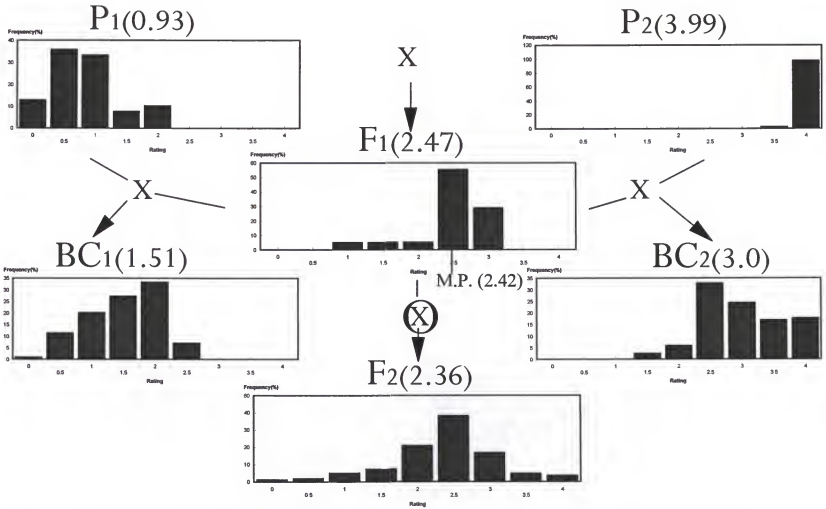


Fig. 3-3. Frequency distributions of ToMoV severity ratings 77 days after planting for P_1, P_2, F_1, BC_1, BC_2, F_2 generations for the cross 719 (derived from accession LA 1932) x 7324 (Spring 1998).

The data sets from the Fall and Spring seasons were compared by analysis of variance, and the differences for the two seasons were not significant ($p=0.91$), although a small season x line interaction was found ($p=0.011$). Analysis of the results for each line, revealed a significant difference ($p<0.01$) between the mean ratings of the BC_2 populations for Fall 1997 (mean disease severity = 3.3) and Spring 1998 (mean disease severity = 3.0). The respective P_1, P_2, F_1, BC_1 and F_2 generations did not differ significantly between the two seasons. As the interpretation of results from both seasons was the same, the two data sets were pooled to improve sampling and simplify the presentation for analysis of ToMoV inheritance (Table 3-3).

Table 3-3. Plant number, mean disease severity rating, variance and standard errors for generations derived from the cross 719 (LA 1932) x Fla. 7324 for Fall 1997 and Spring 1998 combined (77 days after inoculation (D.A.I.) began).

Generation	Number	Mean	Variance	Std. error
P_1 (719)	73	0.73	0.42	0.08
BC_1	233	1.47	0.48	0.05
F_1	78	2.49	0.24	0.06
F_2	789	2.33	0.80	0.03
BC_2	238	3.17	0.44	0.04
P_2 (7324)	77	3.99	0.01	0.01

Frequency distributions for the pooled data set showed the mean of the F_1 population was skewed slightly to the susceptible side of the mid-parent value.

The BC_1 population mean was between the P_1 and F_1 means, and the BC_2 population mean was between the F_1 and P_2 means (Fig 3-4).

The F_2 population segregated continuously, with its mean (2.33) near the mid-parent value (2.36). Collectively, the data indicated that the distribution of the means was mostly additive. Only 5% of the F_2 plants were in the highest resistance categories (0-1), indicating that more than one gene controlled ToMoV resistance. The data had an acceptable fit at p=0.05 to the additive/dominance model using the joint scaling test for the combined data (χ^2 [d.f. 3]= 6.83, p=0.1-0.05) indicating that additive and dominance effects account for most of the genetic variation (Table 3-4).

Table 3-4. Adequacy of the additive-dominance model for combined data from Fall 1997 and Spring 1998.

Generation	Observed mean	Expected mean	Squared deviation	Chi-square value
P_1	0.73	0.62	0.010	1.77
BC_1	1.47	1.51	0.002	1.03
F_1	2.49	2.4	0.009	2.97
F_2	2.32	2.35	0.001	0.63
BC_2	3.17	3.20	0.001	0.42
P_2	3.99	3.99	0.000	0.01
				Total=6.83

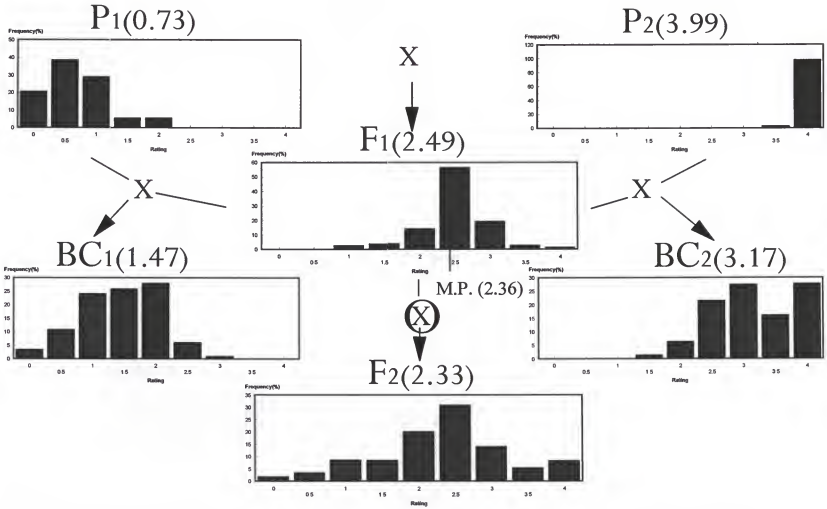


Fig. 3-4. Frequency distributions of ToMoV severity ratings 77 days after planting for P_1, P_2, F_1, BC_1, BC_2, F_2 generations for the cross 719 (derived from accession LA 1932) x 7324 (Fall 1997 and Spring 1998 data combined).

The results suggest that epistasis does not have a significant effect on the variation and homozygous x homozygous (I), heterozygous x homozygous (j) and heterozygous x heterozygous (l) interactions were insignificant. Estimates of the additive and dominance effects were made, and the variation was found to be mainly additive, with a small dominance effect (Table 3-5). Additive effects were significant ($p<0.001$), but no significant dominant effects were found ($p>0.1$). Broad-sense heritability and narrow-sense heritability were high (0.95 and 0.87) indicating that the majority of the genetic variation was caused by additive gene action. Environmental variation was low supporting the high narrow-sense heritability estimate, and an estimate of k (number of effective factors) was calculated and indicated the involvement of 2.29 effective factors.

Table 3-5. Estimates of components of variation, heritability and effective factor number for 719 (LA 1932) a Fla. 7324 cross for combined data from Fall 1997 and Spring 1998.

Variable	Data
Additive variance	-1.68
Dominance variance	0.09
Environmental variance	0.22
Broad-sense heritability	0.95
Narrow-sense heritability	0.87
Effective factor number	2.29

Discussion

Preliminary ToMoV inheritance experiments made in Fall 1996 and Spring 1997 with resistance derived from LA 1932 and LA 1938 (data not included) suggested the involvement of more than one gene. The F_2 populations segregated continuously with a small percentage of plants (10.47% and 8.52 % respectively) in the high resistance category. However, analysis of data was suspect in these experiments due to a small BC_2 population in Fall 1996, and excessive variation of disease ratings in the F_1 population in Spring 1997.

The segregation in Spring 1997 was associated with segregation of the *sp* gene (Silvey, 1974) in the F_1 population. In Fall 1997 and Spring 1998 mean disease severity was shown to be significantly higher in *sp* plants (3.04) than in *sp*+ plants (2.05). The Spring 1997 data were ignored as the resistant parent was heterozygous (*sp/sp+)* resulting in an increased F_1 variation caused by segregation of *sp*. Although resistant *sp* plants were identified, none were in the highest resistance catergory (0, 0.5), indicating that the *sp* locus may be linked to ToMoV resistance.

The experiments highlighted the importance of large BC_1, BC_2 and F_2 populations. Inheritance experiments in Fall 1997 and Spring 1998 used a homozygous *sp*+ resistant breeding line derived from *L. chilense* accession LA 1932, and the plants were rated using whole and intermediate scores (0, 0.5, 1, 1.5

etc.) to better estimate the variation in the populations.

Estimates of broad-sense heritability were high, suggesting that the phenotypic responses to ToMoV were based primarily on the genotype, and the narrow-sense heritability was also high, suggesting that the genetic component of variation was primarily additive. In Fall 1997, an unrealistic estimate of narrow-sense heritability was calculated (Table C-3). However, the method used to calculate this value (Warner, 1952) assumed that the additive variation in the F_2 population was equal to or lower than the sum of the additive variation in the backcross populations. The relatively high F_2 variation caused by a large number of susceptible plants could have resulted in a high numerator in the equation and an inflated estimate of narrow-sense heritability. Alternatively, the variation in the disease severity ratings of the backcross populations may have been low in this season, resulting in a lower than expected denominator in the equation. In Spring 1998, the F_2 variation was lower and realistic estimates of heritability were calculated (Tables C-2 and C-3)

The information obtained was useful in determining the number of generations of inbreeding that need to be made between backcrosses to *L. esculentum*, and the population sizes that need to be planted. If two major additive genes were involved, only 6.3% of the plants were likely to contain the two homozygous genes. Other genes may also influence ToMoV resistance, but

to a lesser extent. This indicated the need for large F_2 populations followed by self-pollinating to ensure all alleles are recovered

Investigations into the inheritance of TYLCV resistance have revealed monogenic (Kasrawi, 1989; Yassin, 1985) and multigenic control; (Pilowsky and Cohen, 1990; Hassan et al., 1984) of resistance. However, the reports have been inconsistent and contradictory (Kasrawi and Mansour, 1990). The inheritance experiments undertaken showed findings similar to one of the studies on *L. pimpinellifolium* where the F_2 progenies indicated that resistance was quantitatively inherited with only a few genes involved (Kasrawi and Mansour, 1990). The inconsistencies reported for TYLCV resistance, suggested that the different sources of geminivirus resistance do not necessarily contain the same resistance genes. The current studies showed no significant indication of dominance in *L. chilense* derived ToMoV resistance genes, which has previously been suggested for TYLCV resistant lines derived from *L. chilense* (Zamir et al., 1994), *L. pimpinellifolium* (Pilowsky and Cohen, 1990) and *L. hirsutum* (Hassan et al., 1984).

Estimates of gene number may have been influenced by linkage, or by regions with different effects on ToMoV resistance. The low estimate of gene number indicated that major genes influence ToMoV resistance, and that it was highly heritable. These results suggested that selection for ToMoV resistance

would be very effective. Hybrids of resistant x susceptible plants have lower levels of resistance than homozygous resistant plants, but heterozygous resistant hybrid varieties would be an improvement over the susceptible varieties available at present. Ultimately, hybrids between two resistant parents would be superior in limiting ToMoV yield reductions.

CHAPTER 4
IDENTIFICATION OF RAPD POLYMORPHISMS WITHIN AND BETWEEN GEMINIVIRUS RESISTANT GENOTYPES DERIVED FROM VARIOUS ACCESSIONS OF *LYCOPERSICON CHILENSE*

Introduction

Tomato mottle virus (ToMoV) is a bipartite geminivirus which has infected Florida tomatoes (*Lycopersicon esculentum* Mill.). It is transmitted by the silverleaf whitefly (*Bemisia argentifolii* Bellows and Perring n. sp.), causing mottling, leaf curling, epinasty, stunting of growth, a reduction in leaf size and a reduction in the yield of the tomato (Abouzid et al., 1992). Epidemics have been responsible for yield losses of up to 20% in the Florida fresh market tomato crop (Schuster, 1992) and no acceptable varieties are commercially available that are resistant to ToMoV.

Tomato yellow leaf curl virus (TYLCV) is a monopartite or bipartite geminivirus with several variants (Kheyr-Pour et al., 1992; Navot et al., 1991; Rochester et al., 1990). First identified in Israel (Cohen and Harpaz, 1966), TYLCV is whitefly transmitted (*Bemisia tabaci* (Genn.), *B. argentifolii*), and is a major disease of tomato throughout the Mediterranean basin, Middle East and Africa (Al-Musa, 1982). Recently in the New World, TYLCV-like disease has been reported in Mexico (Brown et al., 1986) and throughout the Caribbean

(McGlashan et al., 1994; Polston et al., 1994). It has also been reported in North America (Grit et al., 1994), and more recently in Southern Florida (Polston et al., 1997).

Several sources of resistance have been identified that are effective against the geminivirus tomato yellow leaf curl (TYLCV), including *Lycopersicon pimpinellifolium* (Jusl.) Mill. (Pilowsky and Cohen, 1974), *Lycopersicon cheesmanii* Riley and *Lycopersicon hirsutum* Humb. and Bonpl.(Kasrawi et al., 1988; Hassan et al., 1984), *Lycopersicon peruvianum* (L.) Mill. (Pilowsky et al., 1989), and *Lycopersicon chilense* Dunal (Zakay et al., 1991). Commercial varieties or lines tolerant to TYLCV show only limited tolerance to ToMoV. Screening of accessions from wild *Lycopersicon* species with ToMoV (Scott et al., 1995; Scott and Schuster, 1991) revealed several resistant accessions, and suggested that *L. chilense* was the best source for ToMoV resistance. Resistance was found in several *L. chilense* accessions, and twelve accessions (LA 1932, LA 1938, LA 1959, LA 1960, LA 1961, LA 1963, LA 1968, LA 1969, LA 2747, LA 2762, LA 2774 and LA 2779) were crossed with tomato. No interspecific hybrids were obtained from LA 1969, LA 2247, LA 2762 and LA 2774. Of the rest, the most promising resistant lines were derived from LA 1932, LA 1938, LA 1961, LA 1968 and LA 2779 (Scott et al., 1995).

Inheritance studies (chapter 3) suggested the involvement of at least two additive genes in ToMoV resistance, with high heritability and a continuous variation in the F_2. When multiple genes control resistance it is important to incorporate all genes of major effect. To ensure that all resistance genes are fixed in a population, several generations of inbreeding may be necessary between backcrosses (modified backcrossing). The incorporation of these genes may be accelerated by using molecular markers to aid their selection. By identifying molecular polymorphisms linked to resistance genes, it should be possible to select plants containing the resistant genes at each backcross, eliminating the need for inbreeding between backcrosses. This would allow for two crosses per year as opposed to modified backcrossing, thus speeding up the breeding process more than twofold.

Several types of molecular markers may be used in a breeding program including: isozymes (Market and Miller, 1959), restriction fragment length polymorphisms (RFLP) (Botstein et al, 1980), random amplified polymorphic DNA (RAPD) (Williams et al., 1990; Welsh and McClelland, 1990), microsatellites (Morgante and Oliveri, 1993), sequence characterized amplified regions (SCAR) (Paran and Michelmore, 1993) and amplified fragment length polymorphisms (AFLP) (Vos et al., 1995; Zabeau, 1993). Molecular markers have been linked to several resistance genes in tomato including *Pto* (Martin et al.,

1991), *Ty-1* (Zamir et al., 1994), *Mi* (Williamson et al., 1994) and *Tm-2* (Motoyoshi et al., 1996).

The objective of this experiment was to identify RAPD polymorphisms present in lines exhibiting ToMoV or TYLCV resistance. Several resistant lines derived from *L. chilense* accessions (LA 1932, LA 1938, LA 1969, LA 2779) were screened in order to identify the maximum number of polymorphisms, and to identify polymorphisms present in some resistant lines but not others. The RAPD polymorphisms from different sources were then compared to identify common genomic differences in lines exhibiting geminivirus resistance. Several approaches were used for screening the resistant lines including: single plant comparisons from backcross inbreds (Wehrhahn and Allard, 1965), comparisons of near isogenic lines (Young et al., 1988), F_2 tail end comparisons (Darvasi and Soller, 1994), and bulked segregant comparisons (Michelmore et al.,1991). By using different techniques it is possible to maximize the number of polymorphisms identified, to gain a more comprehensive set of markers for linkage to resistance genes.

Materials and Methods

Breeding lines Fla. 7060 (7060) and Fla 7324 (7324) were used as susceptible *L. esculentum* controls for comparisons with resistant lines derived

from accessions of *L. chilense*. Seven ToMoV resistant lines (930670 (670), 960719 (719), 960724 (724), 960744 (744), 97E406S (406), 940699-14 (699), 97E121F (121) (Table B-1)) and two TYLCV resistant lines TY-52 (Hebrew University, Israel) and 97E124F (124) (derived by backcross selection for ToMoV resistance from TYLCV resistant *chiltylc* 94 population provided by H. Laterrot, INRA, France) were used to identify polymorphic RAPD bands. Lines 719, 724 and 744 were derived from *L. chilense* accession LA1932, lines 670, 699 and 406 were derived from *L. chilense* accession LA 1938, lines TY-52 and 124 were derived from *L. chilense* accession LA 1969, and line 121 was derived from *L. chilense* accession LA 2779.

Seeds were sown in wood flats containing Black Beauty spent coal (Real Minerals Div., Highland Ind.) and transplanted at the cotyledon stage to styrofoam trays (3.8 cm^3 cells) where they were grown to the three-leaf stage (approximately 2 weeks). A colony of viruliferous whitefly (*B. argentifolii*) was maintained on the dwarf tomato 'Florida Lanai' in a controlled temperature room at 25^{0}C. Six weeks prior to the inoculation experiments viruliferous whitefly numbers were increased by adding five additional uninfested plants with 7-10 leaves to screened cages. Each plant was fertilized weekly with 118ml of a solution containing 4 gm of 20-20-20 N-P_2O_5-K_2O soluble fertilizer per liter of water. Plants were inoculated for 14 days in a whitefly-proof greenhouse using one source plant for

every 512 seedlings in a screened chamber prior to field planting.

Seedlings were planted in 20 cm high, 76 cm wide beds of EauGallie fine sand that were spaced on 152 cm centers, and plants were spaced 45cm apart within rows. The beds were previously fumigated with 67% methyl bromide: 33% chloropicrin at 239 kg/ha, and covered with polyethylene mulch. Standard fertilization and seepage irrigation practices were used (Hochmuth et al., 1988), and pesticides were used to control foliar bacterial and fungal pathogens according to standard practices. The plants were staked and single tied twice during the season.

Whiteflies were moved to the field with the plants to allow secondary field inoculation, and plants were rated for ToMoV symptoms 35, 56 and 77 days after inoculation began (Table 2-1). DNA was extracted from new leaves (Doyle and Doyle, 1990), and stored at -20°C. Random sequences of DNA were amplified (Williams et al., 1990) using single 10mer oligonucleotide primers (Biotechnology Laboratory, University of British Columbia) in a modified PCR buffer (0.5 M Tris pH 8.3, 10 mM $MgCl_2$, 10 mM Tartrazine, 14% w/v Ficoll), and sequences were separated by gel electrophoresis on a 1.2% agarose gel. DNA was amplified with *Taq* polymerase and 0.2 nM dNTPs for 45 cycles in a Stratgene thermocycler. Each cycle consisted of 60 seconds at 94°C, 60 seconds at 35°C and 90 seconds at 72°C. Gel banding patterns were visualized under ultra-violet light after staining

with ethidium bromide (4 ng/ml), and gel sequences were recorded on Type 55 Polaroid film.

Single plant comparisons. Comparisons were made between five progeny of an F_5 line segregating for resistance (670). Random sequences of DNA from the five plants and a susceptible control (7060) were amplified using 800 arbitrary 10-mer oligonucleotide primers, and amplified sequences were compared by gel electrophoresis. Polymorphic loci were identified between the most resistant plant (670-5), and the least resistant plant (670-8) within the line. Polymorphic loci were also identified between 670 and 7060, the susceptible control.

Bulked sister line comparison. DNA was isolated from a susceptible control (7324) and DNA was bulked from several plants within F_4 lines 719 (resistant) and 724 (intermediate resistance). Random sequences of DNA from 7324, 719 and 724 were amplified using 800 arbitrary 10mer oligonucleotide primers, and amplified sequences were compared by gel electrophoresis. Polymorphic bands were identified between the susceptible control 7324 and the sister lines 719 and 724. Polymorphic loci were identified between the resistant line 719, and the intermediate line 724.

F_2 tail-end comparisons. Line 699 was crossed with 7324 and hybrid seed were obtained. The F_1 was self-pollinated to obtain an F_2 population segregating

for ToMoV resistance genes using seed collected from several plants. An F_2 population of 400 plants was planted in a randomized block design with 4 blocks. The five most susceptible plants identified 77 days after inoculation were bulked (699S) and the five most resistant plants (699R) were all bulked from the F_2 population. The susceptible control 7324, and the bulked DNA samples of the F_2 tail ends were amplified using 800 arbitrary 10mer oligonucleotide primers, and the amplified sequences were compared.

Near isogenic line comparisons (TYLCV resistant/susceptible). Comparisons were made between two near isogenic lines TY-52 and TY-50. One with a partially dominant gene (*Ty-1*) providing resistance against TYLCV, and the other a TYLCV susceptible line ($Ty\text{-}1^+$) (Zamir et al., 1994). DNA was isolated from both lines, and amplified using 800 arbitrary 10mer oligonucleotide primers. The amplified sequences were compared to identify polymorphisms present in the TYLCV resistant line *(Ty-1* region), that were absent in the susceptible line, and vice versa.

Comparison of LA 1969 and LA 2779 derived lines. Two lines with resistance to ToMoV were screened for polymorphic RAPD markers. Line 121 was a resistant selection derived from LA 2779, and 124 was a resistant selection derived from a LA 1969 accession of *L. chilense* (it was initially selected for TYLCV and subsequently selected for ToMoV resistance), with TYLCV tolerant

varieties 'TyKing' and 'Fiona' also in the pedigree. DNA was isolated from all plants, amplified using 600 arbitrary 10mer oligonucleotide primers, and amplified sequences were compared by gel electrophoresis. Polymorphic loci were identified between the two resistant lines and the susceptible control 7324.

Comparison of polymorphisms between different sources. All lines analyzed were derived from *L. chilense* accessions LA 1932, LA 1938, LA 1969 or LA 2779. Polymorphisms were identified from all of the lines, and common polymorphisms were scored in order to identify genetically common regions between the resistant lines. Due to inconsistencies associated with weaker amplifications, the procedures were repeated on two occasions to identify only the clear, repeatable polymorphic bands.

Results

Single plant comparisons. Of the 800 oligonucleotide primers used for amplification, 665 provided sequence amplification, of which 144 showed polymorphisms (Fig. 4-1). When the most resistant line (670-5) was initially compared to susceptible 7060, 111 polymorphisms were identified. Following the second amplification, a total of 49 clear coupling (+) and repulsion (-) polymorphisms were identified between 670-5 and 7060 (35 (+) polymorphisms, 14 (-) polymorphisms).

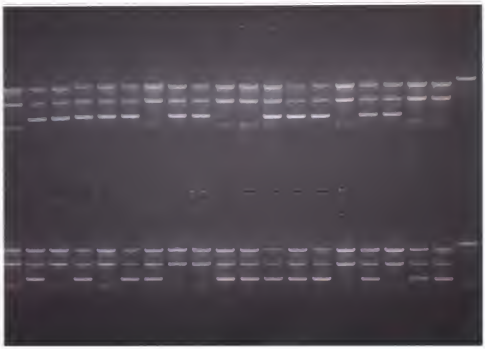


Fig. 4-1. Typical polymorphic RAPD marker. Lane 1, *L. esculentum*; lanes 2-19 polymorphic RAPD polymorphism p058 segregating in an F_2 population derived from LA 2779; lane 20, control amplification.

The within line comparison between 670-5 (most resistant) and 670-8 (least resistant) revealed that 27 of the 35 (+), and 11 of the 14 (-) polymorphisms were present in 670-8. Four additional polymorphisms were identified between 670-8 and 7060 (2 (+) and 2 (-)). The primers providing polymorphisms were designated with arbitrary numbers (p001- p037 (+), and p101- p116 (-)).

Bulked sister line comparisons. Of the 800 oligonucleotide primers used for amplification, 719 provided sequence amplification. A total of 60 polymorphic bands were identified, of which 36 were determined to be clear and repeatable (29 (+) and 8(-)). The resistant sister line (719) accounted for 26 of the coupling (+) polymorphisms. Three of the repulsion (-) polymorphisms were found in both the susceptible control and the intermediate line (724). Fifteen polymorphisms were identified between 719 and the 670 lines (p005, p009, p015, p016, p021, p024, p026, p036, p037, p104, p106, p109, p112, p113, p115) suggesting that LA 1932 and LA 1938 derived lines have common polymorphic regions. Four polymorphic loci were identified with the same primers as 670, but showed different band migrations (p006, p010, p017, p026). The remaining primers identifying new polymorphic loci were designated arbitrary numbers (p042 - p055 (+), p59 and p119 - p120 (-)).

F_2 tail-end comparisons. Of the 800 oligonucleotide primers used, 718 sequence amplifications were observed. A total of 44 (33 (+) and 11 (-)) repeatable polymorphisms were found. The polymorphisms had all been previously identified in the single plant comparisons, indicating association of the identified polymorphisms with ToMoV resistance.

Near isogenic line comparisons (TYLCV resistant/susceptible). Of the 800 oligonucleotide primers used, 684 sequence amplifications were observed, of

which 34 were polymorphic. A total of 23 polymorphisms were clear and repeatable (17 (+) and 6(-)), of which 16 had been previously identified (12 (+), 4(-)), 15 with identical band migration, and one with a different band migration (p025)). The six newly discovered polymorphisms, were designated arbitrary numbers (p038 - p041 (+) and p117- p118 (-)).

Comparison of LA 1969 and LA 2779 derived lines. Of the 600 oligonucleotide primers used, 550 amplified sequences were observed. A total of 33 clear and repeatable polymorphisms were observed (28 (+) and 5 (-)), of which 25 had been previously identified. Of the newly identified polymorphisms, five were common to both lines (p056, p057, p058, p060, p061), two showed different migrations (p059, p063), and one was exclusive to line 111 (p062). The eight newly identified polymorphisms previously unidentified were designated arbitrary numbers (p056-p063 (+)).

Comparison of polymorphisms between different sources. Polymorphisms were identified for the resistant lines screened (Tables 4-1, 4-2), and primers were identified that amplified common polymorphisms in lines derived from different *L. chilense* sources.

Table 4-1. Sources of coupling polymorphisms identified (x=common polymorphism, xx and xxx = common polymorphisms with different migrations).

Source	Line	Primer	Poly.No..	LA1932'	LA1938	LA1969T	LA1969L	LA2779
LA1938	670-5	42	p001		x	x		
LA1938	670-5	53	p002		x	x		x
LA1938	670-5	64	p003		x	x		x
LA1938	670-5	91	p004		x			
LA1938	670-5	110	p005	x	x			
LA1938	670-5	112	p006	xx	x			
LA1938	670-5	116	p007		x		x	x xx
LA1938	670-5	123	p008		x	x		x
LA1938	670-5	131	p009	x	x		x	x
LA1938	670-5	137	p010	xx	x			
LA1938	670-5	148	p011		x	x		x
LA1938	670-5	152	p012		x		xxx	
LA1938	670-5	158	p013		x	x		x
LA1938	670-5	169	p014	xx	x		x	
LA1938	670-5	197	p015	x	x			
LA1938	670-5	211	p016	x	x			
LA1938	670-5	236	p017	xx	x	x		x
LA1938	670-5	302	p018		x xx	x		x
LA1938	670-5	309	p019		x			
LA1938	670-5	371	p020		x			
LA1938	670-5	395	p021	x	x	xx	x	x
LA1938	670-5	400	p022		x			
LA1938	670-5	452	p023		x	x		
LA1938	670-5	462	p024	x	x			
LA1938	670-5	527	p025		x	xx		
LA1938	670-5	538	p026	x xx	x			
LA1938	670-5	555	p027		x	x		
LA1938	670-5	564	p028		x			
LA1938	670-5	572	p029		x			
LA1938	670-5	621	p030		x	x		x
LA1938	670-5	630	p031		x			
LA1938	670-5	648	p032		x			

Table 4-1. cont...

Source	Line	Primer	Poly.No..	LA1932[z]	LA1938	LA1969T	LA1969L	LA2779
LA1938	670-5	697	p033		x	x	x	x
LA1938	670-5	768	p034		x			
LA1938	670-5	788	p035		x			
LA1938	670-8	354	p036	x	x			
LA1938	670-8	389	p037	x	x		x	x
LA1969	TY-52	305	p038	xxx		x	xx	x
LA1969	TY-52	351	p039			x		x
LA1969	TY-52	410	p040			x		x
LA1969	TY-52	550	p041			x		x
LA1932	719	61	p042	x				
LA1932	719	102	p043	x			x	x
LA1932	719	301	p044	x				
LA1932	719	316	p045	x			x	
LA1932	719	330	p046	x				
LA1932	719	333	p047	x				
LA1932	719	348	p048	x				
LA1932	719	365	p049	x				
LA1932	719	374	p050	x				
LA1932	719	423	p051	x				
LA1932	719	504	p052	x				
LA1932	719	570	p053	x				
LA1932	719	579	p054	x				
LA1932	719	625	p055	x			x	
LA2779	1358-Bk	142	p056				x	x
LA2779	1358-Bk	144	p057				x	x
LA2779	1358-Bk	151	p058				x	x
LA2779	1358-Bk	264	p059	x			xx	x
LA2779	1358-Bk	265	p060				x	x
LA2779	1358-Bk	308	p061				x	x
LA2779	1358-Bk	405	p062					x
LA1969	124-Bk	213	p063				x xx	xx

[z] *Lycopersicon chilense* accessions LA 1932 (719, 724), LA 1938 (670-5, 670-8, 699, 406), LA1969T (TY-52), LA1969L (124), LA 2779 (121).

Table 4-2. Table of repulsion (-) polymorphisms identified (x=common polymorphism):

Source	Line	Primer	Poly. No.	LA1932[a]	LA1938	LA1969T	L1969L	LA 2779
LA1938	670-5	42	101		x	x		
LA1938	670-5	102	102		x			
LA1938	670-5	115	103		x			
LA1938	670-5	116	104	x	x			
LA1938	670-5	128	105		x			
LA1938	670-5	177	106	x	x			
LA1938	670-5	244	107		x	x		x
LA1938	670-5	302	108		x			
LA1938	670-5	387	109	x	x		x	
LA1938	670-5	432	110		x	x		x
LA1938	670-5	484	111		x			
LA1938	670-5	599	112	x	x			x
LA1938	670-5	600	113	x	x			x
LA1938	670-5	611	114		x	x		x
LA1938	670-8	135	115	x	x			
LA1938	670-8	283	116		x			
LA1969	TY-52	370	117			x	x	
LA1969	TY-52	657	118			x	x	
LA1932	719-Bk	35	119	x				
LA1932	719-Bk	268	120	x				

[a] *Lycopersicon chilense* accessions LA 1932 (719, 724), LA 1938 (670-5, 670-8, 699,406), LA1969T (TY-52), LA1969L (124), LA 2779 (121)

A total of 46 polymorphic loci were present in lines derived from the different sources (35 (+) and 11 (-)), and the common polymorphisms between two or more of the lines were identified (Table 4-3). Lines derived from LA 1932 had 20 polymorphisms exclusive to that accession (18 (+), 2(-)) and lines derived from LA 1938 had 18 polymorphisms exclusive to that accession (12 (+), 6 (-)). No common polymorphisms were identified between the LA 1932 derived lines and the TYLCV resistant line TY-52, suggesting that the *Ty-1* region is not present in these lines. The majority of the common polymorphisms identified were present in TY-52 and the LA 1938 and LA 2779 derived lines. Polymorphisms common to lines derived from three or more different accessions of *L. chilense* were also identified (Table 4-4).

Table 4-3. Unique (on diagonal) and common coupling (+) and repulsion (-) polymorphic bands from lines derived from *L. chilense* accessions LA 1932, LA 1938, LA 1969, LA 2779.

Accession	LA 1932[z]	LA 1938	LA 1969 T	LA1969 L	LA 2779
LA 1932	18 (+), 2(-)	9(+), 7(-)	0(+), 0(-)	6(+), 1(-)	5(+), 2(-)
LA 1938	-	12 (+), 6(-)	12(+), 4(-)	6(+), 1(-)	13(+), (5)
LA 1969 T	-	-	0 (+), 0(-)	1(+), 2(-)	13(+), 3(-)
LA 1969 L	-	-	-	4 (+), 0(-)	12(+), 0(-)
LA 2779	-	-	-	-	1(+), 0(-)

[z] LA 1932 (719, 724), LA 1938 (670-5, 670-8, 699,406), LA1969T (TY-52), LA1969L (124), LA 2779 (121).

Table 4-4. Common polymorphisms identified from three or four *L. chilense* accessions.

L. chilense accession (LA)[z]	No. of common polymorphisms	Polymorphism no.
1932, 1969L, 2779	1	p043
1938, 1969T, 2779	10	p002,p003,p008,p011,p013, p017,p018,p030,p107,p114
1932, 1938, 2779	2	p112,p113
1932, 1938, 1969L	1	p109
1938, 1969L, 2779	1	p007
1932, 1938, 1969L, 2779	3	p009,p021,p037
1938, 1969T, 1969L, 2779	1	p033

[z] LA 1932 (719, 724), LA 1938 (670-5, 670-8, 699,406), LA1969T (TY-52), LA1969L (124), LA 2779 (121)

The polymorphism data indicated that a common region was present in lines derived from LA 1938, LA 1969T (TY-52) and LA 2779. The lines derived from accession LA 1932 did not have any common polymorphisms with LA 1969T (TY-52), but did have regions in common with LA 1938, LA 1969L (124), and LA 2779 derived lines. One polymorphism (p033) was common to all ToMoV resistant lines, but is not present in the TYLCV resistant line.

Common polymorphisms existed between the ToMoV resistant lines and the susceptible control. Polymorphisms also existed between resistant lines indicating that [1] more than one region was present in ToMoV resistant lines, and

[2] that different regions may be influencing resistance in different lines. The lines bred for ToMoV resistance contain polymorphisms not present in the TYLCV resistant line (TY-52), and LA 1932 derived lines do not appear to have the *Ty-1* gene, indicating that ToMoV resistance is not accounted for by *Ty-1*. Lines derived from LA 1938 and LA 2779 have common polymorphisms with TY-52, indicating that *Ty-1* or a region near *Ty-1* is present in these lines.

Discussion

Several approaches may be used to identify polymorphic markers for linkage to genes of interest and different types of markers are suited to different situations. In this study, RAPD markers were used because they were cost effective, easily identified, and could be used to screen the large populations commonly employed in plant breeding programs. However, RAPD markers can be unreliable, having poor clarity and repeatability, normally lacking co-dominance (preventing the identification of heterozygotes), and requiring additional linkage studies to find their chromosomal location.

To avoid some of these problems, several approaches were made to identify polymorphisms, including F_2 tail-end comparisons, bulk comparisons and near isogenic line comparisons. A large number of polymorphisms were identified and confirmed for clarity and repeatability on two occasions. Coupling (+) and

repulsion (-) polymorphisms were also identified so that repulsion linked markers could be combined to identify heterozygotes. To determine which polymorphisms are most likely to be linked to ToMoV resistance, polymorphisms common to lines derived from different *L. chilense* accessions were compared, to identify regions conserved in the different resistance lines.

The methods for identification of polymorphisms differed in their effectiveness. Single plant comparisons were effective because they were made between F_5 plants with high levels of homozygosity. Comparison of near isogenic lines and sister lines also provided clear polymorphisms due to the high level of homozygosity. The comparisons of F_2 tail ends were less successful because the bulking of plants at the tail-ends did not guarantee that all resistant genes were present/absent in all plants within each bulk. One plant heterozygous for a resistance gene can easily be included in the susceptible bulk, preventing identification of that region. As more than one gene appeared to be involved in ToMoV resistance, it was essential to identify polymorphic loci using more than one approach, in order to identify all regions linked to resistance genes.

The results indicated that several polymorphisms identified in the studies fell into different groups. These groups were based [1] on the accession of *L. chilense* from which they were derived and [2] the comparisons with lines derived from different accessions. The polymorphisms indicated that exclusive regions

exist in lines derived from LA 1932 and LA 1938, and a region in line 121 and 124 is common. Polymorphisms common between different lines also suggested that resistance genes from different sources may be the same or located in similar chromosomal regions. The line TY-52 has the resistance gene *Ty-1* on the short arm of chromosome 6. This gene reportedly provides resistance against TYLCV (Zamir et al., 1994), but does not provide resistance against ToMoV (Scott, personal communication). Polymorphisms identified in TY-52 near isogenic lines are also present in lines derived from *L. chilense* accessions LA 1938 and LA 2779, suggesting that these lines contain the *Ty-1* gene (or a gene near this chromosomal location).

To determine the effect of the regions linked to the polymorphisms identified, it is necessary to compare the segregation of the polymorphisms with the segregation of disease resistance. Analysis of F_2 populations can provide information to link polymorphic markers to one another, and to regions involved with ToMoV resistance.

CHAPTER 5
LINKAGE OF RAPD POLYMORPHISMS TO TOMATO MOTTLE VIRUS (ToMoV) RESISTANCE GENES

Introduction

Tomato mottle virus (ToMoV) is a bipartite geminivirus which first appeared in Florida in the late 1980s (Kring et al., 1989). It is transmitted by the silverleaf whitefly (*Bemisia argentifolii* Bellows and Perring n. sp.) and infects the Florida tomato (*Lycopersicon esculentum* Mill.) crop, causing mottling, leaf curling, epinasty, stunting of growth, a reduction in leaf size and a reduction in yield of the tomato (Abouzid et al., 1992). Sporadic epidemics have been responsible for yield losses of up to 20% in the Florida fresh market tomato crop (Schuster, 1992).

The chemical control of whitefly has been difficult, as whitefly (particularly *B. argentifolii*) quickly develop resistance against many organophosphorus and pyrethroid insecticides, and they are also present in several different developmental stages (Al-Musa, 1986). In 1994 the systemic insecticide imidacloprid was introduced, and this has resulted in control of both ToMoV and TYLCV. Imidacloprid binds to the soil around the plants root system, and becomes effective against immature whitefly after a three week period. While

selection pressure for imidacloprid resistance is high, there are no current reports of resistant whitefly in Florida (Cahill et al., 1996). ToMoV and TYLCV resistant varieties are desirable due to the possibility of insect resistance to pesticides. Resistance genes effective against the geminivirus tomato yellow leaf curl virus (TYLCV), have been reported from several *Lycopersicon* species including: *Lycopersicon pimpinellifolium* (Jusl.) Mill. (Kasrawi, 1989), *Lycopersicon peruvianum* (L.) Mill. (Pilowsky and Cohen, 1990) and *Lycopersicon chilense* (Zamir et al., 1994). The resistance gene *Ty-1* was identified in a line derived from *L. chilense* accession LA 1969 and was mapped to the short arm of chromosome 6 on the centromeric side of RFLP marker TG 97 (Zamir et al., 1994). Although some TYLCV tolerant lines are available (Pilowsky et al., 1989; Zamir et al., 1994)), no ToMoV resistant varieties are currently available.

Screening of wild *Lycopersicon* species accessions with ToMoV (Scott et al., 1995; Scott and Schuster, 1991), revealed several resistant accessions, and suggested that *Lycopersicon chilense* (Dunal) was the best source of ToMoV resistance. Resistance was found in several *L. chilense* accessions, and twelve of these accessions (LA 1932, LA 1938, LA 1959, LA 1960, LA 1961, LA 1963, LA 1968, LA 1969, LA 2747, LA 2762, LA 2774 and LA 2779) were crossed with tomato. Hybrids were obtained from all but LA 1969, LA 2747, LA 2762 and LA

2774 and of those tested the most promising resistant lines were derived from LA 1932, LA 1938, LA 1961, LA 1968 and LA 2779 (Scott et al., 1995).

When multiple genes control resistance, it is important to incorporate all genes of major effect and to ensure that all resistance genes are fixed in a population. Several generations of inbreeding may be necessary between backcrosses (modified backcrossing) to achieve this result. The incorporation of these genes may be accelerated by using molecular markers to aid their selection. By identifying molecular polymorphisms linked to resistance genes, it should be possible to select plants containing the resistant genes at each backcross, eliminating the need for inbreeding between backcrosses. This would allow for two crosses per year as opposed to modified backcrossing, thus speeding up the breeding process more than twofold.

Linkage of markers to genes of interest (Sax, 1923; Morgan, 1911) allows the selection of desirable genotypes using phenotypic traits, or more recently, molecular markers. The tomato is ideally suited to linkage studies as it has a short generation time, a perfect flower, and a large number of phenotypic mutations (Stevens and Rick, 1986). Several types of molecular markers may be used in a breeding program including: isozymes (Market and Miller, 1959), restriction fragment length polymorphisms (RFLP) (Botstein et al, 1980), random amplified polymorphic DNA (RAPD) (Williams et al., 1990; Welsh and McClelland, 1990),

microsatellites (Morgante and Oliveri, 1993), sequence characterized amplified regions (SCAR) (Paran and Michelmore, 1993) and amplified fragment length polymorphisms (AFLP) (Vos et al., 1995; Zabeau, 1993).

Molecular markers have been linked to several resistance genes in tomato including bacterial speck resistance (*Pto)* (Martin et al., 1991), tomato yellow leaf curl resistance *(Ty-1)* (Zamir et al., 1994), nematode resistance *(Mi)* (Williamson et al., 1994) and tomato mosaic virus resistance *(Tm-2)* (Motoyoshi et al., 1996). Comprehensive isozyme and RFLP linkage maps have also been created for tomato (Tanksley et al., 1992; Tanksley and Rick, 1980), providing information on chromosomal locations of morphological and molecular markers.

Inheritance studies indicated that at least two additive genes were involved in ToMoV resistance (chapter 3), and screening of resistant lines for polymorphic RAPD markers revealed homologous polymorphisms common to ToMoV resistant lines derived from *L. chilense* accessions LA 1932, LA 1938, LA 1969 and LA 2779 (chapter 4). A total of 81 polymorphisms were identified (61 coupling markers, 20 repulsion markers), 19 of which were present in at least three lines derived from different accessions. Polymorphisms were identified in lines derived from *L. chilense* accessions LA 1938 and LA 2779 that were exclusive to the lines, and others were present in the TY-52 (*Ty-1*) and TY-50 (*Ty-1*+) isolines.

Tomato plants have either a determinate (*sp*) growth habit or an indeterminate (*sp*+) growth habit (Silvey, 1974). ToMoV resistant lines derived from *L. chilense* have exhibited less severe symptoms in indeterminate plants than in determinate plants (Scott et al., 1995), indicating possible linkage of ToMoV resistance to the *sp* locus on the long arm of chromosome 6. The recessive *c*-gene, which causes the potato leaf instead of the usual cut-leaf phenotype (Rick and Butler, 1956), and the *B*-gene, which causes beta-carotene to be the dominant fruit pigment (Rick and Butler, 1956), have been previously mapped close to the *sp* locus on chromosome 6 (Fig. 5-1) (Weide et al., 1993; Tanksley et al., 1992) and segregate in F_2 populations of ToMoV resistant lines. The *c*-gene segregated only in determinate F_2 populations derived from *L. chilense* accession LA 1932. The objective of these experiments was to gain information on the chromosomal location of ToMoV and TYLCV resistant regions, and to link the different regions to one another and morphological marker genes. The possible linkage of these morphological markers to ToMoV resistance was investigated, and the RAPD polymorphisms identified were also used to investigate possible linkages between each other, morphological traits, and ToMoV resistance in segregating F_2 populations.

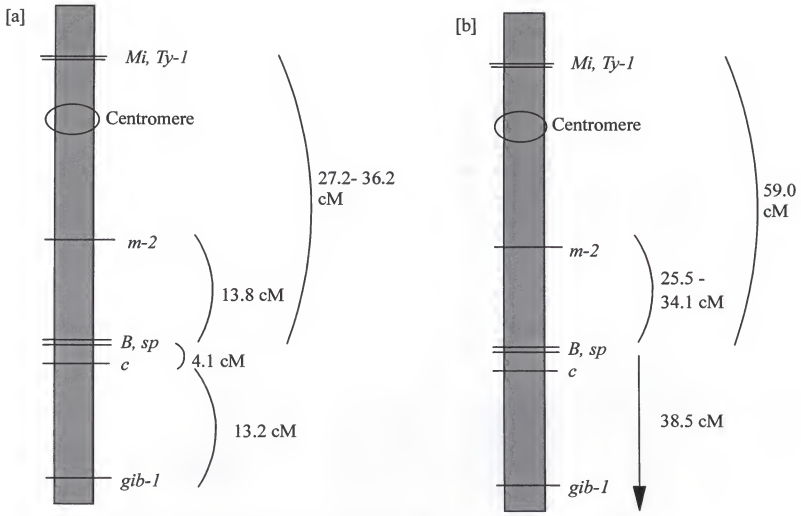


Fig. 5-1. Linkage maps of chromosome 6 modified from [a] Weide et al., 1993, [b] Tanksley et al., 1992.

Materials and Methods

ToMoV resistant lines 930670-5 (719), 980432 (719), and 960729 (729) were derived from *L. chilense* accessions LA 1938, LA 1932 and LA 1932 respectively (Table B-1). Crosses were made between the resistant lines and susceptible breeding lines (670-5 x Fla. 7171 (7171), 719 x Fla. 7324 (7324), and 729 x Fla. 7613 (7613)), and F_1 plants were self-pollinated to provide F_2 populations segregating for ToMoV resistance.

ToMoV susceptible and resistant lines were sown in wood flats containing Black Beauty spent coal (Real Minerals Div., Highland Ind.), and transplanted at the cotyledon stage to styrofoam trays (3.8 cm^3 cells) where they were grown to the three leaf stage. A colony of viruliferous whitefly (*B. argentifolii*) was maintained on the dwarf tomato 'Florida Lanai' in a controlled temperature room at 25^0C. Six weeks prior to the inoculation experiments, viruliferous whitefly numbers were increased by adding five additional uninfested plants with 7-10 leaves to screened cages. Each plant was fertilized weekly with 118ml of a solution containing 4 gm of 20-20-20 N-P_2O_5-K_2O soluble fertilizer per liter of water. Plants were inoculated in a greenhouse for 14 days using one source plant for every 512 seedlings prior to field planting.

Seedlings were planted in 20 cm high, 76 cm wide beds of EauGallie fine sand that were spaced on 152 cm centers. Plants were spaced 45 cm apart within

rows, and were staked and single tied to the stakes twice during the season. The beds were previously fumigated with 67% methyl bromide: 33% chloropicrin at 239 kg/ha and covered with black polyethylene mulch. Standard fertilization and seepage irrigation practices were used (Hochmuth et al., 1988), and pesticides were used to control foliar bacterial and fungal pathogens according to standard practices.

Whitefly were moved to the field with the plants to allow secondary field inoculation, and plants were rated for ToMoV severity 35, 56 and 77 days after inoculation began (D.A.I.) according to the rating scale (Table 2-1). DNA samples were extracted from new leaves (Doyle and Doyle, 1990), and stored at -20°C. Random sequences of DNA were amplified (Williams et al., 1990) using 10-mer oligonucleotide primers (Biotechnology Laboratory, University of British Columbia) in a modified PCR buffer (0.5M Tris pH 8.3, 10mM $MgCl_2$, 10mM Tartrazine, 14% w/v Ficoll), and sequences were separated by gel electrophoresis on a 1.2% agarose gel. DNA was amplified with *Taq* polymerase and 0.2nM dNTPs for 45 cycles in a thermocycler (Stratagene), where each cycle consisted of 60 seconds at 94°C, 60 seconds at 35°C and 90 seconds at 72°C. Gel banding patterns were visualized under ultra-violet light after staining with ethidium bromide (4 ng/ml), and gel sequences were recorded on Type 55 Polaroid film.

Morphological markers and polymorphic RAPD markers were identified and scored. Mean disease severity ratings were calculated for each line, and genotypes were tested for significant associations with mean disease severity using "SAS for Windows" (SAS Institute, 1997) general linear model analysis. Linkage analyses were made between segregating phenotypic and molecular markers using Joinmap 1.3 (Stam, 1993).

Segregation of the potato leaf gene (*c*-gene) with ToMoV resistance. ToMoV resistant line 729 (*sp*, *c*) was crossed with 7613 (*sp*, *c*+), and the F_1 plants were self-pollinated to provide an F_2 population segregating for ToMoV resistance and potato leaf. A total of 91 F_2 plants were rated for ToMoV resistance (Table 2-1) and potato leaf. The plants were rated on a scale of 1 to 4 (*cc* (1), *cc*+(2/3), *c*+*c*+(4)), as they did not show the typical recessive potato-leaf phenotype, and mean disease severity ratings were tested for significant associations with ToMoV resistance. The segregation of the 7 most reliable polymorphic markers (p006, p010, p014, p024, p042, p050, p059) was determined, and mean ToMoV disease severity ratings were calculated for ratings made 35, 56 and 77 D.A.I..

Segregation of the self pruning (sp) gene with ToMoV resistance. ToMoV resistant line 719 (*sp*+) was crossed with susceptible line 7324 (*sp*), and the F_1 plants were self pollinated to obtain seed for a segregating F_2 population. Mean

disease severity ratings of 277 F_2 plants were calculated for ratings made 35, 56 and 77 D.A.I., and plants were scored for *sp* and *sp*+.

Segregation of polymorphic RAPD markers in an LA 1932 derived F_2 population. ToMoV resistant line 719 (*sp*+) was crossed with susceptible line 7324 (*sp*), and F_1 plants were self-pollinated to provide seed for F_2 populations that were grown in Fall 1997 and Spring 1998 . A total of 367 plants segregating for ToMoV resistance and polymorphic markers were rated and scored. Disease severity ratings were taken 35, 56 and 77 D.A.I., and the segregations of the six most reliable polymorphic markers were scored (p005, p014, p015, p021, p042, p119). Linkage between the markers was determined using Joinmap 3.1 (Stam, 1993).

Segregation of polymorphic RAPD markers in an LA 1938 derived F_2 population. ToMoV resistant line 670-5 (*sp*+) was crossed with susceptible line 7171 (*sp*), and F_1 plants were self-pollinated to provide seed for an F_2 population of 80 plants segregating for ToMoV resistance and polymorphic markers. Disease severity ratings were taken 35, 56 and 77 D.A.I., and the segregation of the 11 most reliable polymorphic markers (p002, p003, p009, p010, p015, p018, p020, p024, p025, p030, p034) was determined.

Construction of linkage map for chromosome 6 based on segregation data from combined F_2 populations. The data from the F_2 populations derived from the three crosses (729 x 7613, 719 x 7324 and 670-5 x 7171) were combined in order to construct a linkage map based on phenotypic and genotypic polymorphisms from populations derived from *L. chilense* accessions LA 1932 and LA 1938. RAPD polymorphisms were linked to each other, and to the known phenotypic markers *sp* and *c* on chromosome 6 using Joinmap 1.3 (Stam, 1993)..

Association of polymorphic regions to ToMoV resistance derived from *L. chilense* accession LA 1932. An F_2 population of 367 plants was screened for primers p042 (+/-), p014(+) and p119(-). Primer p042 (+/-), which amplified co-dominant polymorphisms, mapped distal to the *c*-gene on the long arm of chromosome 6. Primers p014 (+) and p119 (-) mapped proximal to the *sp*-gene on the long arm of chromosome 6. Mean disease severity ratings taken 35, 56 and 77 days after inoculation began were grouped according to the 9 possible genotypes identified with the polymorphisms, and Duncan's multiple range test was used to determine means groupings of the genotypes to determine association with ToMoV resistance.

Results

Segregation of the potato-leaf gene (*c*-gene) with ToMoV resistance. The population segregated 71 ($cc/cc^+/c^+c$):16(c^+c^+), which fits the chi-square goodness of fit test at $p \geq 0.05$ for a 3:1 ratio (χ^2 (d.f.1)=2.02, $p>0.1$). The plants did not exhibit the typical recessive phenotype for potato leaf, and some inaccuracy was involved in the late season determination of potato leaf which may have led to some *cc* homozygotes being rated as heterozygotes. ToMoV disease severity was rated for each plant, and segregations of polymorphic RAPD markers were identified. Associations between ToMoV ratings and the *c*-gene were tested by comparing ratings of *cc* with *c*+*c*+ (Table 5-1), and linkage of the RAPD polymorphisms to the c-gene was established (Table 5-2).

Table 5-1. Mean disease severity of an F_2 population segregating the *c*-gene.

Genotype	Number	Rating time[z]		
		35 days	56 days	77 days
cc (1)	16	1.44 b[zz]	2.12 a	1.91 c
Het. (2)	26	1.65 ab	2.48 a	2.1 bc
Het. (3)	32	2.03 ab	2.77 a	2.66 a
c+c+ (4)	13	2.12 a	2.81 a	2.50 ab

[z] Rating time in days after inoculation began.
[zz]Duncan's mean separation in columns by Duncan's multiple range test at $p \leq 0.05$.

Table 5-2: Linkage of polymorphic RAPD markers relative to the potato leaf locus (*c*-gene)in an F_2 population derived from the cross 729 (LA 1932) x 7613.

Polymorphism No.	Primer No.	Map Distance (cM)
p042 (+/-)	UBC 61	40.5
p006-2	UBC 112	40.0
p010-2	UBC 137	39.2
p050	UBC 374	34.9
c-gene	-	0
p059	UBC 264	4.1
p024	UBC 462	25.4

The plants rated as homozygous for potato-leaf (*cc*) and cut-leaf (*c+c+*), had significantly different mean disease severity at the 35 and the 77 day ratings (Table 5-1), indicating that the homozygous potato leaf plants have a lower level of disease severity when infected with ToMoV. When combined, the plants rated as heterozygotes have a mean disease severity intermediate to that of the homozygotes for all of the ratings, suggesting that the effective region associated with ToMoV resistance is additive. Association of the phenotypic marker causing potato-leaf suggests the involvement of chromosome 6 in ToMoV resistance.

The marker order was determined for the 729 x 7613 derived F_2 population, which segregated for polymorphism p024 present in ToMoV resistant lines derived from *L. chilense* accession LA 1938. Six polymorphisms were mapped to the c-gene with relaxed LOD scores to determine the order of linkage groups (Table 5-2). All markers were linked to the c-gene, again indicating the

importance of chromosome 6 in ToMoV resistance. Two markers were oriented with the LA 1938 markers (p059, p024), and four markers were oriented the side of LA 1932 identified markers (p006-2, p010-2, p042 (+/-), p050).

Segregation of the self-pruning (*sp*) gene with ToMoV resistance. The 277 F_2 plants tested segregated for sp, 203 were indeterminate, fitting the 3:1 expected ratio (χ^2 (d.f. 1) = 0.436, p>0.1). Association of the *sp*+ locus with ToMoV resistance was tested, and indeterminate plant habit was found to have a significantly lower mean disease severity for all three ratings (Table 5-3).

Table 5-3. Mean disease severity rating of determinate (*sp*) and indeterminate (*sp*+) plants in an F_2 population derived from a ToMoV resistant line (719 (*sp*+)) x susceptible breeding line (7324(*sp*)).

Plant Habit	Number	Rating (D.A.I)[z]		
		35	56	77
Indeterminate (*sp*+)	203	1.48b[zz]	1.95b	3.05b
Determinate (*sp*)	74	2.02a	2.86a	2.05a

[z] Rating time in days after inoculation began.
[zz] Duncan's mean separation in columns by Duncan's multiple range test at $p \leq 0.05$.

The significant association of this locus to ToMoV resistance indicates the involvement of chromosome 6. The resistance linked to this locus also appears to be effective throughout the season, having its largest effect later in the season 77 days after inoculation began.

Segregation of polymorphic RAPD markers in an LA 1932 derived F_2 population. When the L.O.D. is relaxed to account for linkages close to 50%, the order of markers on a multi-locus map can be determined (Table 5-4). Four markers were linked closely to the *sp*/*sp*+ locus (p015, p119, p021, p014), and two were linked at a distance (p005, p042). The markers were found to map to both sides of this locus. The results suggested the importance of one region close to the *sp*-gene, and a second region that is present at a distance from the *sp*-gene.

Table 5-4. Linkage of polymorphic RAPD markers relative to the *sp*/*sp*+ locus in an F_2 population derived from the cross 719 (derived from LA 1932) x 7324.

Polymorphism No.	Primer No.	Map Distance (cM)
p015	UBC 197	22.0
p119	UBC 35	4.8
sp/sp+	-	0
p021	UBC 395	1.7
p014	UBC 169	2.4
p005	UBC 110	60.4
p042	UBC 61	63.6

Segregation of polymorphic RAPD markers in an LA 1938 derived F_2 population. All markers tested were linked with a relaxed L.O.D. score of 1.0 to allow for linkages of close to 50%, allowing the best fitting order on a multilocus map to be determined. The two groups of markers were linked, and mapped relative to the *sp* locus (Table 5-5). The 11 RAPD markers and the *sp* locus

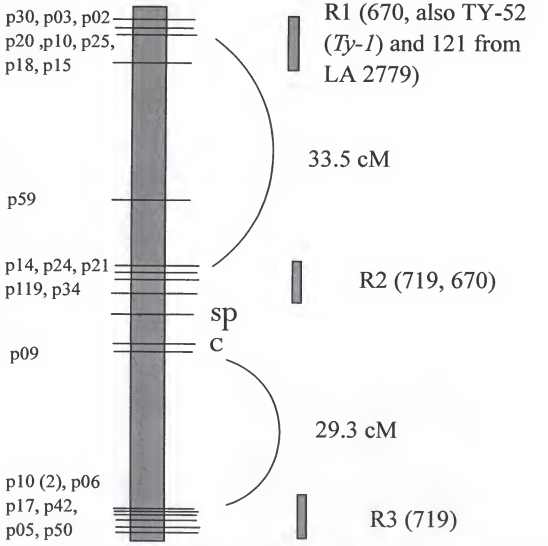
comprised two linkage groups, 3 polymorphisms linked to *sp*+, and eight linked to one another. The relaxed linkage limits allowed the three polymorphisms linked to the *sp* locus (p009, p034, p024) to be associated with the eight other polymorphisms (p015, p018, p025, p010, p020, p002, p003, p030). The association of these two groups indicates the presence of two regions on chromosome 6, that have been retained in a resistant line derived from *L. chilense* accession LA 1938. Polymorphism p015 was analyzed in the 719 F_2 population, at a distance of 22 cM from the *sp* locus (Table 5-3), compared to a distance of 42.7 cM in this population.

Table 5-5. Linkage of polymorphic RAPD markers relative to the *sp* locus in an F_2 population derived from the cross 670-5 (LA 1938) x 7171.

Polymorphism No.	Primer No.	Map Distance (cM)
p030	UBC 621	51.5
p003	UBC 64	49.7
p002	UBC 53	49.7
p020	UBC 371	49.6
p010	UBC 137	49.4
p025	UBC 527	49.2
p018	UBC 302	49.1
p015	UBC 197	42.7
p024	UBC 462	15.3
p034	UBC 768	13.5
p009	UBC 131	5.7
sp/*sp*+	-	0.0

The discrepancy in map distances was most likely due to population size, as only 80 plants were examined for p015 in the 670-5 derived F_2 population. All markers mapped to one side of the *sp* locus in the 670-5 x 7171 cross, suggesting that the 719 x 7324 population had a region not present in the 670-5 derived population. The 670-5 derived population also had a region the same side of *sp* as p015, but at a greater distance. These results suggested that at least three regions on chromosome 6 were conserved in ToMoV resistant lines derived from *L. chilense* accessions LA 1932 and LA 1938.

Construction of a linkage map for chromosome 6 based on segregation data from combined F_2 populations. The segregation data from the three F_2 populations were combined, and a linkage map of chromosome 6 revealed 3 regions with clusters of markers in ToMoV resistant lines that were not present in susceptible control lines (Fig. 5-2). The *c*-gene mapped to the telomeric side of the *sp*-locus and was closely linked (4.7cM).



Primer	cM
p030	0
p003	1.8
p002	1.8
p020	2.0
p010	2.1
p025	2.3
p018	2.3
p015	9.0
p059	25.4
p014	35.8
p024	36.2
p021	36.5
p119	36.7
p034	37.3
sp gene	41.5
c gene	46.2
p009	48.2
p010(2)	75.5
p006	77.5
p017	78.6
p042	78.9
p005	79.4
p050	80.1

***Fig. 5-2*:** Linkage map of chromosome 6 based on linkage data from three F_2 populations.

The three regions R1, R2 and R3 had 7, 5 and 6 RAPD polymorphisms respectively, and 3 RAPD polymorphisms mapped to intermediate positions (p015, p059, p009). Regions R1 and R2 were separated by 33.5 cM, and regions R2 and R3 were separated by 38.2 cM, with a total map distance of 80.1 cM. Four polymorphisms in region R1 (p030, p003, p002, p018) were present in the TYLCV resistant line TY-52 (*Ty-1* gene) (Zamir et al., 1994), and 3 of the polymorphisms (p030, p003, p002) were found in line 111 derived from *L. chilense* accession LA 2779 (chapter 6). Only polymorphisms identified from LA 1932 derived lines were found in region R3, and no polymorphisms identified in TY-52 were identified in either of the regions R2 and R3. Three of the 5 polymorphisms identified in region R2 (p014, p024, p021) were common to both LA 1932 and LA 1938 derived ToMoV resistant lines. Two repulsion markers were mapped (p042(-) and p119), both repulsion markers were identified in LA 1932 derived ToMoV resistant lines.

Association of polymorphic regions to ToMoV resistance derived from *L. chilense* accession LA 1932. Co-dominant polymorphisms from regions R2 and R3 were identified using the repulsion linked RAPD markers p014 (+) and p119 (-) (0.9cM apart), and the co-dominant RAPD marker p042, respectively. The mean disease ratings were calculated for each of the nine possible genotypes and the results are illustrated (Fig. 5-3, Table 5-6).

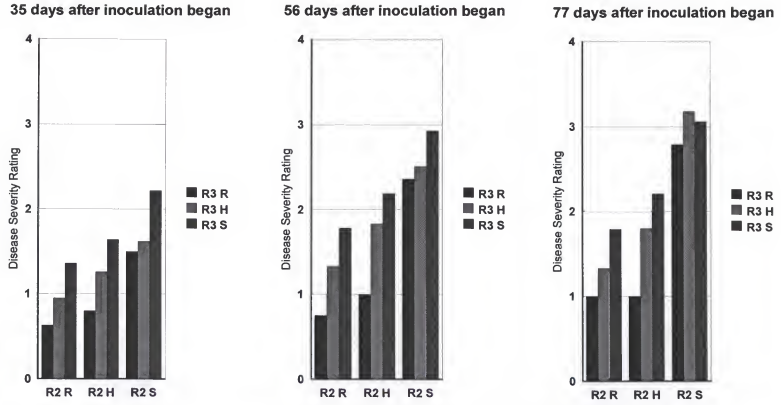


Fig. 5-3. Effect of region R2 and R3 genotypes on ToMoV disease severity in an F2 population with resistance derived from LA 1932. R = resistant, H = heterozycous, S = susceptible.

Table 5-6. Effect of regions R2 and R3 on ToMoV resistance.

Regions	Marker Genotype	Rating[z]		
		35 days	56 days	77 days
[1] R2 (++), R3 (++)	p014,p119,p042(+),	0.63c[zz]	0.75d	1.00d
[2] R2 (++), R3 (+ -)	p014,p119,p042(+),p042(-)	0.95bc	1.33cd	1.33cd
[3] R2 (++), R3 (- -)	p014,p119,p042(-)	1.36a-c	1.78bc	1.79bc
[4] R2 (+-), R3 (+ +)	p014,p042(+)	0.80bc	1.00d	1.00d
[5] R2 (+-), R3 (+ -)	p014,p042(+),p042(-)	1.26bc	1.83bc	1.80bc
[6] R2 (+-), R3 (- -)	p014,p042(-)	1.64ab	2.19ab	2.21b
[7] R2 (- -), R3 (+ +)	p119,p042(+)	1.50a-c	2.36ab	2.79a
[8] R2 (- -), R3 (+ -)	p119,p042(+),p042(-)	1.62ab	2.51ab	3.18a
[9] R2 (- -), R3 (- -)	p119,p042(-)	2.22a	2.93a	3.06a

[z] Rating time in days after inoculation began.
[zz] Duncan's mean separation in columns by Duncan's multiple range test at $p \leq 0.05$.

Plants homozygous for the R2 genotype ([3]) had effective resistance throughout the season and had significantly lower levels of ToMoV disease severity at the 56 and 77 day ratings than plants missing both genotypes ([9]). Plants homozygous for the R3 genotype ([7]) had lower levels of disease severity earlier in the season (35 day after inoculation began) when compared to [9], although this difference was not significant. Plants heterozygous for both genotypes ([5]) had significantly lower ToMoV disease severity than [9] for all ratings. Plants homozygous for both genotypes ([1]) were the most resistant plants, and were significantly better than [9] throughout the season.

Heterozygotes were intermediate to the homozygotes for both genotypes, suggesting that the effects were additive (Fig. 5-3).

Discussion

Potato leaf is caused by the *c*-gene which is recessive and located close to the *sp* locus on the long arm of chromosome 6 (Rick and Butler, 1956). However, its exact location is ambiguous since it has been mapped on the centromeric side (Tanksley et al., 1992) of the *sp* locus, and on the telomeric side (Kush and Rick, 1968; Weide et al., 1993). The *c*-gene has been described as a recessive gene; however, the trait inherited from *L. chilense* showed an intermediate leaf shape which was presumed to be heterozygous. Designation of the *L. chilense c*-gene was not always clear from the phenotype, which may have been due in part to ratings that were taken later in the season when potato leaf is less clear than at the seedling stage. Potato leaf was only observed in determinate plants, suggesting an epistatic interaction preventing expression of this phenotype in indeterminate plants. Potato leaf was observed in determinate lines derived from *L. chilense* accession LA 1932, it was also observed in TYLCV resistant lines received from Laterrot (derived from *L. chilense* LA 1969 and 'TyKing'), and in a TYLCV tolerant landrace 'TyKing'. There was not a clear association of the c-gene to resistance, as it was unlikely to be the only gene involved with the resistance in

this population. The underlying genotype at the *c* locus could not be determined accurately due to the ambiguities of classification of the leaf shape phenotype.

It was previously noted that indeterminate plants had greater ToMoV resistance than determinate plants (Scott and Schuster, 1991). Analysis of the segregation of the *sp* gene with ToMoV resistance revealed a significant association of indeterminate plants (*sp*+) with ToMoV resistance, with a larger influence on resistance later in the season. To determine linkage of polymorphic RAPD markers to this locus, one determinate and two indeterminate (*sp*+) ToMoV resistant lines were crossed to susceptible lines to produce F_2 populations segregating at *sp*/*sp*+ and the RAPD markers. Not all RAPD markers identified in chapter 4 were suitable due to inconsistencies in amplification caused primarily by the crude DNA preparation used for screening F_2 populations.

Region 1, which encompassed 7 polymorphisms (p030, p003, p002, p020, p010, p025,p018), mapped 39.2 cM from the *sp* locus. Four of these markers were identified in the TYLCV resistant line TY-52 (p030, p003, p002, p018), indicating that this region may be homologous to the *Ty-1* gene. The *Ty-1* gene was mapped close to the nematode resistance locus (*Mi*) on the short arm of chromosome 6, which has previously been mapped at distances of 36.2 cM, 59.0 cM and 56.7 cM from the *sp* locus (Weide et al., 1993; Tanksley et al., 1992; Khush and Rick, 1968). The location of this group of markers, and the presence

of four of these markers in the TYLCV resistant line indicates that this region is homologous or identical to the *Ty-1* region. Further evidence was provided by Zamir (personal communication) when he screened lines derived from *L. chilense* accessions LA 1932 and LA 1938. RAPD markers from region R1 amplified polymorphisms in the LA 1938 lines, but not the LA 1932 derived lines. RFLP markers linked to *Ty-1* were used to screen DNA from the ToMoV resistant plants, and the RFLP markers were identified in the LA 1938 derived lines in the heterozygous form, but were not located in the LA 1932 derived lines (Zamir, personal communication).

Markers within 5.3 cM of *sp* were located in ToMoV resistant lines derived from accessions LA 1932 and LA 1938, indicating the involvement of a factor effective in ToMoV resistance very close to *sp*. The close linkage to this locus explains some of the difficulties involved in breeding determinate lines with high levels of ToMoV resistance. A repulsion marker was mapped into this linkage group, allowing the determination of heterozygous genotypes from this region.

A third region R3 was identified near the telomere on the long arm of chromosome 6, and it was mapped 29.3 cM from the *c*-gene. The region R3 was exclusive to lines derived from accession LA 1932 and was associated with potato leaf plants. A further phenotypic abnormality associated with many lines containing this region has been small and very sparse vines. The *gib-1* gene is an

induced mutation deficient in endogenous gibberellins and exhibits phenotypes including extreme dwarfism, reduced germination and abnormal flower development (Jacobsen et al., 1994). Previous linkage studies have mapped the *gib-1* gene 13.2 cM or 34.2 cM from the *c*-gene (Weide et al., 1993; Tanksley et al., 1992), that is, in approximately the same location as region R3. The map distance of *gib-1* from the *c*-gene has a large disparity in previous studies, although the latter group suggests suppression of recombination in this region (van Wordragen et al., 1996).

The results herein suggest that an *L. chilense* derived gene involved in the gibberellin pathway might cause similar symptoms to the *gib-1* mutation, and be allelic or closely linked. The *gib-1* like symptoms were only observed in determinate plants, suggesting an epistatic interaction is occurring preventing expression in indeterminate plants. The linkage to these traits has been broken in ToMoV resistant lines derived from LA 1932 (Scott, personal communication). Of the RAPD markers identified in this region, polymorphism p042 was co-dominant, and five other markers were dominant.

To determine the effects of these regions on ToMoV resistance, F_2 populations based on the cross 719 x 7324 were analyzed. The regions R2 and R3 segregated in the F_2 population with ToMoV resistance, and heterozygotes of both regions were identified using repulsion linked markers (R2), and a co-dominant

marker (R3) to determine their genotypes. The results suggested that two additive genes are effective in line 719, which was also determined by statistical analysis in the inheritance studies (chapter 3).

The use of these markers for developing commercial varieties is yet to be determined and the linkages between ToMoV resistant genes and deleterious traits must first be broken. It is likely that region R2 will be the most difficult region to select due to its close linkage to the *sp* locus. To breed all three regions into a line, it may be necessary to select for several cross-overs to reduce linkage drag. The identification of RAPD markers for these regions could provide valuable information enabling the adoption in breeding approaches previously not possible because crosses can be selected that will ensure all resistance genes will be maintained in the progeny.

The RAPD polymorphisms identified could be used to accelerate breeding for ToMoV resistant lines by using backcross breeding rather than the present method of modified backcrossing. The polymorphisms that provide markers in several different lines and have close linkage to ToMoV resistance, but no linkage to deleterious traits, could be used to produce SCAR markers. The effect of region R1 could also be examined to determine the effect it has on ToMoV resistance, alone, or combined with regions R2 and R3. Combinations of the different regions should also be tested for resistance to TYLCV.

CHAPTER 6
YELLOW MOSAIC SYMPTOM RESPONSE TO TOMATO MOTTLE VIRUS INOCULATION IN A GENOTYPE DERIVED FROM *LYCOPERSICON CHILENSE* ACCESSION LA 2779

Introduction

Tomato mottle virus (ToMoV) first appeared in Florida in the late 1980s (Abouzid et al., 1992; Kring et al., 1989). It is a whitefly-transmitted (*Bemisia argentifolii* Bellows and Perring n. sp.) geminivirus and infects the Florida tomato (*Lycopersicon esculentum* Mill.) crop causing mottling, leaf curling, epinasty, stunting of growth, and a reduction in yield and leaf size (Polston et al., 1993).

In screening wild accessions for ToMoV resistance, bright yellow mosaic symptoms were observed in some accessions of *Lycopersicon chilense (Dunal), Lycopersicon peruvianum* (L.) Mill., *Lycopersicon hirsutum* Humb. and Bonpl., and *Lycopersicon pennellii* (Corr.) which were different from the mottling normally associated with ToMoV infection (Fig. 6-1) (Scott and Schuster, 1991). Subsequent screening of F_2 populations based on one cross of the ToMoV resistant lines derived from *L. chilense* accession LA 2779, revealed plants segregating with either bright yellow mosaic symptoms (Fig.6-1), typical ToMoV symptoms (Fig.1-4), or intermediate symptoms which were a less extreme expression on the bright yellow mosaic response (Fig.6-2)

Fig. 6-1. Yellow mosaic response to ToMoV infection in tomato.

Fig. 6-2. Intermediate response to ToMoV infection in tomato.

The atypical responses to ToMoV infection were observed and selected against for several seasons, but remained present in a line derived from LA 2779. This line had been backcrossed to a ToMV resistant line, suggesting that the response might be derived from alleles at the *Tm-2* locus. This was further supported by the appearance of bright yellow mosaic symptoms in breeding lines which were resistant to fusarium crown rot (incited by *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL)) and ToMV (Scott, personal communication).

The bright yellow mosaic reaction to ToMoV infection appeared at varying times following inoculation of progeny from 971358-4 (Table B-1), usually appearing 2-3 weeks after inoculation, but sometimes not until late into the growing season. Plants exhibiting the yellow symptoms later in the season did not have typical symptoms of ToMoV preceding this response, and no obvious stunting of growth or reduction in yield was observed. Tomato mosaic virus (ToMV) resistance is provided by the allelic genes *Tm-2* and *Tm-2a* on chromosome 9 (Ganal et al., 1989), and PCR markers for this region have been previously identified (Motoyoshi et al., 1996; Ohmori et al., 1995). The bright yellow response to ToMoV infection was observed in lines with known resistance to ToMV, however; the delayed response was only observed in lines bred for ToMoV resistance (Scott, personal communication). The yellow mosaic pattern was examined, to establish the genetic basis of the atypical response to ToMoV

infection, and its possible relationship to ToMV resistance.

Linkage of molecular markers to phenotypic traits of interest allows selection of the phenotype, based on linked genetic information. Several markers may be used to determine linkage including: isozymes (Market and Miller, 1959), restriction fragment length polymorphisms (RFLP) (Botstein et al., 1980), random amplified polymorphic DNA (RAPD) (Williams et al., 1990; Welsh and McClelland, 1990), microsatellites (Morgante and Oliveri, 1993), sequence characterized amplified regions (SCAR) (Paran and Michelmore, 1993) and amplified fragment length polymorphisms (AFLP) (Vos et al., 1995; Zabeau, 1993). Molecular markers have been linked to several resistance genes in tomato including: bacterial speck resistance (*Pto*) (Martin et al., 1991), tomato yellow leaf curl tolerance (*Ty-1*) (Zamir et al., 1994), nematode resistance (*Mi*) (Williamson et al., 1994), tomato mosaic virus resistance (*Tm-2*) (Motoyoshi et al., 1996) and provide information of considerable use in breeding programs.

To better characterize the region responsible for the bright yellow mosaic symptoms, plants segregating for this character in populations of ToMoV-resistant lines derived from accession LA 2779 were inoculated and examined. The RAPD technique was used to identify polymorphic markers linked to the bright yellow mosaic disease response, and the relationship with tobacco mosaic virus (ToMV) resistance was examined.

Materials and Methods

A colony of viruliferous whitefly (*B. argentifolii*) was maintained on the dwarf tomato 'Florida Lanai' in a controlled temperature room at 25^{0}C. Six weeks prior to the inoculation experiments, viruliferous whitefly numbers were increased by adding five additional uninfested plants with 7-10 leaves to screened cages. Each plant was fertilized weekly with 118ml of a solution containing 4 gm of 20-20-20 N-P_2O_5-K_2O soluble fertilizer per liter of water. ToMoV resistant line 121 (971358-4) was derived from *L. chilense* accession LA 2779, and segregated yellow mosaic symptoms, typical symptoms and intermediate symptoms in response to inoculation with ToMoV. Plants from within line 121 were examined in comparison with the heat tolerant breeding line Fla. 7324 (7324). Eight selections from the ToMoV resistant breeding line 121 were made that showed bright yellow mosaic (2 selections), intermediate (4 selections) and typical chlorotic mottle symptoms (2 selections). The susceptible control and the eight selections were sown in wood flats containing Black Beauty spent coal flats (Real Minerals Div., Highland Ind.) on 9 January, 1998 and transplanted at the cotyledon stage to styrofoam trays (3.8 cm^3 cells) on 23 January. The trays were moved to a screened chamber for inoculation at the 2-3 leaf stage of development, and inoculated for 14 days with whitefly from the infested 'Florida Lanai' plants on 2 February using one source plant for every 512 seedlings.

Seedlings were planted on 16 February in 20 cm high, 76 cm wide, beds of EauGallie fine sand that were spaced on 152 cm centers and plants were spaced 45 cm apart within rows. The beds were previously fumigated with 67% methyl bromide: 33% chloropicrin at 239 kg/ha, and covered with polyethylene mulch. Standard fertilization and seepage irrigation practices were used (Hochmuth et al., 1988), and pesticides were used to control foliar bacterial and fungal pathogens according to standard practices. Three plots of each line were planted, two plots contained 10 plants each and the third plot contained 20 plants. Plants were staked, and single tied to the stakes. Whitefly were moved to the field with the plants to allow secondary field infection, and plants were rated for ToMoV symptoms at 21 day intervals 35, 56 and 77 days after inoculation began (Table 2-1). No whitefly pesticides were applied.

DNA was extracted from new leaves (Doyle and Doyle, 1990), and stored at -20°C. Random sequences of DNA were amplified (Williams et al., 1990) using 10mer oligonucleotide primers (Biotechnology Laboratory, University of British Columbia) in a modified PCR buffer (0.5M Tris pH 8.3, 10mM $MgCl_2$, 10mM Tartrazine, 14% w/v Ficoll), and sequences were separated by gel electrophoresis on a 1.2% agarose gel. DNA was amplified with *Taq* polymerase and 0.2nM dNTPs for 45 cycles in a Stratgene thermocycler, each cycle consisted of 60 seconds at 94°C, 60 seconds at 35°C and 90 seconds at 72°C. Gel banding

patterns were visualized under ultra-violet light after staining with ethidium bromide (4ng/ml), and gel sequences were recorded on Type 55 Polaroid film.

DNA was amplified using 400 oligonucleotide primers from plants exhibiting yellow symptoms (Yellow), intermediate symptoms (Int) and typical chlorotic mottle (Mottle) in response to ToMoV inoculation. The banding patterns were compared with those of 7324 on an agarose gel, and polymorphisms were identified. Lines were screened with the polymorphic markers identified from the line 121 comparisons. Five SCAR markers were obtained that were linked closely to the *Tm-2* locus on chromosome 9 (Motoyoshi et al., 1996). Plants were screened for presence of the *Tm-2* gene with the SCAR markers, and association between polymorphic RAPD markers was identified using Joinmap 1-3 (Stam, 1993).

Results

Of the 400 primers used for amplification, 12 resulted in clear and repeatable polymorphisms (Table 6-1). Five of these were present in the majority of the 121 plants and had been previously identified (p002, p003, p018, p033, p040). These were mapped to chromosome 6 at a location homologous to the TYLCV resistance gene *Ty-1* (Chapter 5). DNA extracted from plants exhibiting yellow mosaic or intermediate symptoms amplified 5 common polymorphisms

(p007-2, p009-2, p039-2, p058, p062). These polymorphisms were not amplified in the 7324 susceptible controls or in line 121 plants exhibiting typical disease symptoms. Polymorphism p062 was co-dominant amplifying a coupling (p062) and repulsion polymorphism (p062-2) at the same locus (Table 6-1). Two repulsion polymorphisms were identified in 7324 and line 121 plants exhibiting intermediate and typical response to ToMoV infection (p121, p122). The coupling and repulsion markers were identified in the plants showing intermediate symptoms, suggesting that they were heterozygous for this locus.

Table 6-1. Polymorphisms identified from Fla. 7324 progeny of line 121 (LA 2779 derived) that segregated typical green symptoms, intermediate symptoms and yellow mosaic symptoms.

Primer	Poly. no	7324	Mottle	Int	Yellow
UBC 53	p002		x	x	x
UBC 64	p003		x	x	x
UBC 302	p018		x	x	x
UBC 697	p033		x	x	x
UBC 410	p040		x	x	x
UBC 116	p007-2			x	x
UBC 131	p009-2			x	x
UBC 351	p039-2			x	x
UBC 151	p058			x	x
UBC 405	p062			x	x
	p062-2	x	x	x	
UBC 389	p121	x	x	x	
UBC 395	p122	x	x	x	

The RAPD polymorphisms previously identified in TY-52 (*Ty-1* gene) and linked to chromosome 6 were not present in all lines tested. Plants exhibiting bright yellow mosaic, intermediate and mottle symptoms all segregated for the chromosome 6 polymorphism. The results indicate that 8 polymorphisms not amplified in TY-52 (*Ty-1*), segregated differently and were not linked to this locus.

The segregation of progeny from the eight selections indicated that the yellow and intermediate symptom response was controlled by one locus (Table 6-2). The two selections of bright yellow mosaic plants had yellow progeny, with two plants remaining completely uninfected until the end of the season. The intermediate selections segregated yellow, intermediate and mottle progeny which fitted a 1:2:1 ratio at $p=0.01$ ($\chi^2= 7.92$ [d.f. 2], $p<0.025$, $p>0.01$) suggesting heterozygosity for this locus. The poor fit to the 1:2:1 ratio could have been caused by mis-diagnosis of some of the intermediates as mottle and/or late onset of yellow/intermediate symptoms. None of the progeny from the mottle selections were yellow or intermediate. The mottle selections showed typical responses to ToMoV inoculation; however, the responses were mild with no completely susceptible plants observed, suggesting the influence of another region(s) on ToMoV resistance.

Table 6-2. Segregation of progeny from eight line 121 (LA 2779 derived) selections that showed yellow (2), intermediate (4) and mottle (2) symptom response to ToMoV infection.

Selection	Yellow	Int	Mottle	Green	Total
Yellow (2)	63	-	-	2	65
Int (4)	32	51	45	-	128
Mottle (2)	-	-	68	-	68

The four plants with intermediate symptom were selected from line 121, and the progeny were screened with 12 polymorphic RAPD markers (Table 6-1). Two linkage groups were identified, the first linkage group (Table 6-3) contained markers (p002, p003, p018, p033, p040) that had been previously identified (chapter 5) and mapped to chromosome 6. These all marked the same region as indicated by a 0 cM map distance. The second linkage group, was unique to line 121 and was linked to the yellow mosaic ToMoV symptom (Table 6-4).

Table 6-3. Linkage of 5 polymorphic markers from line 121.

Polymorphism	Primer	Map Distance (cM)[z]
p002	UBC 53	0
p003	UBC 64	0
p018	UBC 302	0
p033	UBC 697	0
p040	UBC 410	0

[z] Determined with Joinmap 1.3.

Table 6-4. Linkage of polymorphic markers from line 121 to yellow mosaic disease response.

Polymorphism	Primer	Map Distance (cM)[z]
Yellow mosaic	-	0
p007-2 (+)	UBC 116	10.0
p039-2 (+)	UBC 351	10.1
p009-2 (+)	UBC 131	10.1
p062 (+)	UBC 405	10.1
p058 (+)	UBC 151	10.1
Tm-2 SCAR	OPB 12	10.1
p062 (-)	UBC 405	10.4
p122 (-)	UBC 395	10.4
p121 (-)	UBC 389	12.0

[z] Determined with Joinmap 1.3.

Five SCAR markers for ToMV were screened, and one of the five (OPB 12) provided a dominant polymorphism. Linkage of the ToMV marker to polymorphisms identified from line 121 and to plants exhibiting the bright yellow mosaic symptoms were determined (Table 6-4) and the markers identified all mapped to within 2 cM of the *Tm-2* locus SCAR. The bright yellow mosaic phenotype mapped 10 cM from the markers, although phenotypic determination of this response may have been inaccurate due to late symptoms incomplete penetrance of the yellow symptoms in highly resistant plants.

Discussion

The bright yellow mosaic and the intermediate symptom responses to ToMoV infection appeared at different times throughout the season, but once identified spread rapidly throughout the plant. Prior to the symptom response, progeny of plants selected for bright yellow mosaic symptoms remained healthy and symptom free even late in the season. If this symptom response could be delayed to late season consistently in breeding lines showing bright yellow and intermediate symptoms, it could prove to be a valuable genotype in breeding for ToMoV resistance.

The bright yellow mosaic and intermediate symptom responses were rapid, systemically infecting the entire plant within several days of first appearing. The

rapid onset of this response in other branches became obvious with yellowing of the veins, followed by movement into the leaf tissue. It was possible that ToMoV infection was being restricted to the vascular tissue, but after prevention of movement into leaf cells broke down in one area perhaps when a threshold level was reached, cell to cell movement of the virus occurred throughout the leaf tissue of the entire plant.

The yellow mosaic and intermediate responses were found to be linked to the *Tm-2* ToMV resistance locus. This could prove useful in breeding work as ToMV is highly infectious, and can be difficult to work with in a breeding program. By inoculating with whitefly viruliferous for ToMoV, it is possible to identify ToMV resistant plants by selecting those showing yellow mosaic symptoms, removing the need for ToMV inoculation. A second disease resistance gene on chromosome 9 (*Frl*) confers resistance to fusarium crown rot and it is linked to *Tm-2* and $Tm\text{-}2^2$ (Vakalounakis et al., 1997; Laterrot and Couteaudier, 1989) at a distance of 5.1 cM (Tanksley et al., 1992). Inoculation with FORL can be difficult due to environmental factors that can alter the disease screening process (Jones et al., 1990). By inoculating lines with ToMoV these environmental limitations can be overcome by selecting for plants with a yellow mosaic response, providing a more accurate screening procedure.

The SCAR OPB-12 was closely linked to *Tm-2*, with a map distance of 0.7 cM (Motoyoshi et al., 1996), and the eight RAPD polymorphisms identified all mapped within 2 cM of this SCAR, suggesting that the polymorphic region identified in the plants exhibiting atypical symptoms was homologous to the *Tm-2* locus on chromosome 9. The yellow symptom response mapped 10 cM from the molecular markers; however, this may have been caused by non-expression of symptoms in some plants. Some plants that were genetically intermediate were rated as having typical symptoms, and were primarily responsible for the linkage distance. It is possible that mis-classification of plant symptom response was caused by late season onset of intermediate symptoms beyond the rating 77 D.A.I., and this may have led to a larger than expected map distance from the *Tm-2* locus. The yellow mosaic symptoms have been observed in ToMV resistant FORL breeding lines and in ToMV resistant breeding lines from Israel and Taiwan, suggesting that alleles at the *Tm-2* locus (*Tm2* or $Tm2^2$) or a very closely linked gene is responsible for the atypical response to ToMoV infection.

To better clarify the effects of ToMoV resistance genes with the onset of this response, lines should be created that contain the regions identified in chapter 5 and the *Tm-2* region. Some ToMoV resistant lines show delayed expression of the yellow symptoms late into the season, whereas ToMV resistant lines not bred for ToMoV resistance express the yellow symptoms within three weeks on

inoculation with ToMoV (data not shown). If the symptom response could be delayed throughout the season, the absence of symptoms observed prior to the response could be of value in creating geminivirus resistant breeding lines.

The delay in symptom response in plants exhibiting yellow and intermediate symptoms is not the same in all lines (data not shown) and appears to be controlled genetically. It would be useful to associate markers with regions contributing to the delay of the yellow mosaic symptoms in ToMV resistant plants. The delay in symptoms may be caused by interaction of ToMoV resistance genes on chromosome 6, or even a ToMoV resistance gene on chromosome 9 linked to the *Tm-2* locus in the lines studied. We are presently following up on the interaction of this region with crosses to plants containing ToMoV regions identified in chapter 5.

CHAPTER 7
SUMMARY AND SUGGESTIONS FOR FUTURE RESEARCH

Tomato mottle virus (ToMoV) is a whitefly transmitted geminivirus infecting Florida tomato crops, causing serious reductions in yield. Sources of resistance to ToMoV have been identified from *Lycopersicon chilense* (Dunal) wild accessions (Scott and Schuster, 1991), and resistance has been introgressed into tomato (Scott et al., 1995). Little is known about the inheritance of the resistance.

The objectives of this study were to determine the most effective procedure to artificially inoculate the ToMoV resistant lines with viruliferous whitefly, to investigate the mode of inheritance of resistance to ToMoV, and to identify molecular markers linked to ToMoV resistance genes. Visual determination of disease severity and mass inoculation with viruliferous whitefly in a screened chamber were used due to the large numbers of plants grown, and secondary field infection helped prevent inaccurate resistance ratings caused by plant escapes during the inoculation procedure.

Inoculation using viruliferous whitefly has not been clearly documented and has frequently been referred to with generic statements in TYLCV papers (Zamir et al., 1994; Zakay et al., 1991) perhaps due to the inconsistencies

involved in the procedure. Vector inoculation is potentially more variable than non-vector inoculation largely because of insect behavior and inability to precisely subject each plant to a uniform density of inoculum. It is not practical to inoculate large plant numbers using biolistic inoculation or agroinoculation, and procedures such as agroinoculation can infect plants resistant to natural infection (Kheyr-Pour et al., 1994). Natural inoculation with viruliferous whitefly can be made in the field (Vidavsky et al., 1998) where large environmental differences cause inconsistent disease pressure. Cages (Pico et al., 1998) are inefficient for inoculating large plant numbers. Mass inoculation in screened chambers has been reported to overcome resistance in some tolerant plants, while inoculation of some plants was not done due to whitefly preference (Pico et al., 1998).

Mass inoculation with viruliferous whitefly in a screened chamber was used, followed by secondary field inoculation. Field inoculation is undesirable as it provides a potential disease pool for local growing districts. To determine the effectiveness of the initial inoculation without secondary infection, whitefly were killed following inoculation using a thiodan spray and an imidacloprid systemic insecticide drench. Early ratings were recorded to determine the number of plant escapes from the initial inoculation, before secondary infection from neighboring plants in the field. The experiments undertaken examined the effects of duration of the inoculation time (I.D.) to develop a mass inoculation procedure in a

screened chamber that avoids plant escapes.

The experiments indicated that a 9 day inoculation was sufficient to obtain a 100% infection without secondary field inoculation, if trays were rotated in the chamber during the inoculation and one source plant was used for every 4 trays (512 seedlings). The experiments also indicated that an extended inoculation time caused a significant breakdown in the resistance of lines. Despite the plant to plant variation observed, the experiments provided sufficient information to recommend a procedure for inoculation.

Future experiments should further examine the inconsistencies involved in inoculation experiments. The range of whitefly number on the plants is not mentioned in most of the literature involving whitefly inoculation even though large variations in whitefly number per plant can occur (Table 2-2). Field inoculation may differ significantly in disease pressure between seasons (Vidavsky et al., 1998), and an inoculation of 48 hours with 15-20 whitefly in insect-proof cages has been suggested (Pico et al., 1998). Cage inoculation could be examined for ToMoV inoculation and compared to inoculation in screened chambers. The experiments in chapter 2 suggested that a screened inoculation of 48 hours is insufficient; however, lower whitefly numbers were involved in these experiments. Future studies should compare not only the effectiveness of cage inoculation with screened mass inoculation, but they should also compare whitefly

number. Control of whitefly number is more consistent in insect-proof cages, although routine scoring of whitefly number and control of source plant number could be used to estimate and control vector numbers in the chamber.

The experiments performed were subject to secondary field inoculation from inoculated adjacent plots. The experiments were not done in another area of the research station to avoid spreading disease to unrelated experimental plots. The inoculated plants should be grown in ToMoV free areas, to better determine the effectiveness of the procedure in the absense of secondary inoculation. It has also been suggested that whiteflies selectively ignore small leaved plants during mass inoculation in a screened chamber (Pico et al., 1998). Whitefly preference could cause inaccurate designation of resistance levels in plants, particularly in lines with wild characteristics. However, large numbers of whitefly have been observed on small leaved plants in the field, and inoculated wild accessions with characteristics including small leaves became infected. Experiments could be made to compare the mean disease severity of lines with small leaves or unusual characteristics including blue/green leaf color when cage inoculated as opposed to the normal inoculation in a screened chamber.

To ensure reliable ratings in experiments, it is important that the inoculation procedure is effective. Inoculation of up to 14 days did not appear to significantly overcome resistance in ToMoV resistant lines and appeared to be

effective causing 100% infection (particularly with secondary field inoculation). Although inoculation of plants for 48 hours (Pico et al., 1998) has been suggested, this may not be sufficient to differentiate the plants with the highest levels of resistance from those with moderate resistance. A 14 day inoculation was used for the experiments in the inheritance and linkage studies, as this was considered suitable for 100% inoculation with minimal breakdown in resistance levels caused by high disease pressure.

Previous inheritance studies on geminivirus resistance have been undertaken only on tomato yellow leaf curl virus (TYLCV) and have produced variable results (Kasrawi et al., 1989; Hassan et al., 1984; Pilowsky and Cohen, 1973) perhaps due to differences in plant material, environmental differences and inconsistent inoculation procedures in different countries of the Middle East (Kasrawi and Mansour, 1990). The current studies suggested that ToMoV resistance in a line derived from *L. chilense* accession LA 1932, involved 2 or more genes, was highly heritable, and the genetic gain was mainly additive. Future inheritance studies could examine lines derived from other accessions, as marker work suggests that genes could be present in some lines that are not present in others (Chapters 4, 5 and 6).

ToMoV resistant lines showing intermediate resistance could also be examined to potentially gain information on the inheritance of individual ToMoV

resistance genes. These experiments could involve generations derived from crosses of lines identified in chapter 5, which have homozygous regions associated with ToMoV resistance. By using lines with known genetic information, it would be possible to test the effectiveness of the Mather-Jinks inheritance analysis.

Experiments should avoid resistant lines with linked morphological characteristics such as the self-pruning (*sp*) gene. The *sp* locus is linked to ToMoV resistance (chapter 5). To avoid possible rating inconsistencies between plants determinate (*sp*) and indeterminate (*sp*+) growth habits, inheritance studies should be based on crosses of plants with the same morphological characteristic (for example, *sp* x *sp*, *sp*+ x *sp*+). An experiment could also be undertaken that avoids secondary field inoculation and involves a lower disease pressure, this could uncover genes that have an effect against ToMoV that is overcome with large whitefly numbers in the field. Identical inheritance studies could also be undertaken for TYLCV resistance. By comparing studies on ToMoV and TYLCV it may be possible to better characterize ToMoV resistance that is effective against TYLCV, and vice versa.

To better characterize the resistance to ToMoV, experiments were undertaken to mark resistance genes using linked molecular polymorphisms. RAPD polymorphisms were identified from ToMoV and TYLCV resistant lines

derived from four different accessions of *L. chilense* (LA 1932, LA 1938, LA 1969 and LA 2779), and polymorphisms common to the different sources were identified. Polymorphisms identified in lines derived from LA 1938 and LA 2779 were common to those identified from the TY-52 (*Ty-1*) / TY-50 (*Ty-1+*) near isogenic lines, suggesting that a region present in some ToMoV resistant plants was homologous to a region previously identified in TYLCV tolerant plants (Zamir et al., 1994). Polymorphisms were also identified that were not present in the TYLCV resistant lines, suggesting that previously unidentified ToMoV resistance genes were present in lines derived from accessions LA 1932, LA 1938 and LA 2779.

Linkage studies on F_2 populations segregating these polymorphisms revealed 3 clusters of polymorphic RAPD markers linked to the morphological markers self-pruning (*sp*) and potato leaf (*c*) on chromosome 6. Two of the regions (R2 and R3) flanked the *sp* locus and the region containing common polymorphisms with the TYLCV tolerant line TY-52 (R1) was mapped 39.2cM from the *sp* locus on the centromeric side. The region common to TY-52 was in a homologous location to the *Ty-1* gene which had previously been mapped 56.9cM from the *sp* locus (Zamir et al., 1994; Tanksley et al., 1993).

Analysis of F_2 populations segregating the regions R2 and R3 indicated significant associations of both regions with ToMoV resistance. Plants

heterozygous for the regions showed intermediate levels of resistance, suggesting that the genes are additive thus supporting the interpretations of the inheritance studies that were made using the same F_2 populations. Future studies could help characterize the effects of the individual regions on ToMoV resistance in F_2 populations. These results could be compared to F_2 populations segregating combinations of the different regions.

Several random amplified polymorphic DNA (RAPD) markers were identified for each region. Further studies could help identify the polymorphisms most closely linked to ToMoV or TYLCV resistance genes. Studies could also identify the markers that are consistent in the largest number of breeding lines. Once the most reliable and universal markers are identified, sequence characterized amplified region (SCAR) markers (Paran and Michelmore, 1993) could be created from clones of the amplified regions. The construction of SCAR markers would allow the amplification of single band polymorphisms which can show a higher reliability than RAPD markers.

Many of the RAPD polymorphisms will be lost during breeding as linkages to undesirable genes are broken. Consequently, it is important to identify the polymorphisms with most potential as resistant lines are backcrossed to commercial breeding lines. If the polymorphisms identified are not linked tightly enough to the resistance genes, the segregations can be used to identify

polymorphisms using different types of molecular markers including amplified fragment length polymorphisms (AFLPs) (Vos et al., 1995) and restriction fragment length polymorphisms (RFLPs) (Botstein et al., 1980).

Plants containing individual ToMoV resistant regions and combined ToMoV resistant regions, can also be inoculated with TYLCV to ascertain the effects of the different ToMoV resistance genes against TYLCV. To help determine whether resistant region R1 is homologous to the *Ty-1* gene, lines segregating this region could be inoculated with TYLCV and compared to the near isogenic lines TY-52 and TY-50. A repulsion polymorphism for region R1 could also provide more information on its association with ToMoV and TYLCV resistance in future studies.

The *sp* gene has been cloned (Pnueli et al., 1998) and is the ortholog of the *cen* gene in Arabidopsis. Overexpression of this gene in transgenic indeterminate plants suppresses the transition of the vegetative apex to a reproductive shoot resulting in a determinate growth habit. If the resistance genes linked very closely to the *sp* locus are necessary, but cannot be bred into a determinate plant by conventional means, it may be possible to overcome this by transforming with the *sp* clone.

Polymorphisms in a fourth region were associated with ToMoV resistant plants derived from accession LA 2779. The region caused atypical disease

symptoms when inoculated with ToMoV and was found in several lines containing alleles at the *Tm-2* locus for tomato mosaic virus (ToMV) resistance. The polymorphisms were linked tightly to a previously identified SCAR marker for *Tm-2* (Motoyoshi et al., 1996), confirming the location of this region on chromosome 9. The onset of the atypical symptoms is delayed in some lines bred for ToMoV resistance through to late season and prior to the onset of this response plants remain disease free. If the yellow mosaic symptom response could be delayed consistently throughout the season, the material could provide the best source of ToMoV resistance identified. Future work could identify the cause of the delay in plants exhibiting the atypical response, and determine whether it is genetic or environmental. The translocation of the virus could be examined to help explain the mode of action of the resistance. Tissue blots taken from different parts of the plant could help explain the rapid onset of the bright yellow mosaic response throughout the plant, and determine whether the virus is present in the symptomless plants prior to the onset of this response. Lines showing the bright yellow and intermediate responses could also be inoculated with TYLCV to examine the effect of this region on TYLCV resistance.

The RAPD polymorphisms identified can be used to accelerate breeding for ToMoV resistant lines by using backcross breeding rather than the present method of modified backcrossing. By selecting the regions associated with

ToMoV resistance after each backcross, experiments can be undertaken selecting for the resistant genotype and horticultural characteristics without the need for inoculating the material. Tests could be made on smaller populations to ensure that linkage between the RAPD markers and the ToMoV resistance genes has not been broken.

APPENDIX A
CHARACTERISTICS AND DISTRIBUTION OF GEMINIVIRUSES.

Table A-1. Characteristics of geminiviruses in the Western Hemisphere (Polston and Anderson, 1997).

Virus	Known distribution	Symptoms in tomato	Other Hosts
Chino del tomate (CdTV)/Tomato leaf crumple virus (TLCrV)	Mexico	Severe leaf curling, leaf rolling, yellow mosaic, stunting, reduced fruit set.	Host range includes 19 species in 4 families.
Pepper huasteco virus (PHV)	Mexico	Vein clearing, leaf distortion, chlorosis, stunting.	*Capsicum annum*
Potato yellow mosaic virus (PYMV)	Guadeloupe, Martinique, Puerto Rico, Trinidad, Tobago, Venezuela	Chlorotic mottle, leaf rolling, leaf distortion	Species of *Datura, Petunia, Nicotiana, Solanum tuberosum*
Serrano golden mosaic virus (SGMV)	Mexico, U.S. (Arizona)	Leaf curling, leaf distortion, stunting	*C.annum, C.frutescens, Datura stramonium*
Sinaloa tomato leaf curl (STLCV)	Mexico	Foliar chlorosis and purpling, leaf curling, shortened internodes	*C.annum, Nicotiana tabacum, Solanum melongena*
Taino tomato mottle virus (TTMoV)	Cuba	Rugosity, leaf deformation and curling, stunting.	*C. annum*
Texas pepper virus (TPV)/Pepper jalapeno virus (PJV)	Mexico, Guatemala, U.S. (Arizona, Texas)	Mosaic, leaf curling, stunting	*C.annum, C.frutescens, D.stramonium*, 4 *Nicotiana* spp. *P. wrightii, N.glauca*
Tomato geminivirus Bz-Ub	Brazil	Yellow mosaic	Not reported
Tomato geminivirus Bz-lg	Brazil	Yellow mosaic	Not reported
Tomato golden mosaic virus (TGMV)	Brazil	Yellow mosaic	*D.stramonium*, 5 *Nicotiana* spp., *Physalis* spp.
Tomato mottle virus (ToMoV)	Puerto Rico, U.S. (Florida, S.Carolina, Tennessee, Virginia)	Chlorotic mottle, leaf curling, stunting, reduced fruit size and number	*Lycopersicon* spp., *Phaseolus vulgaris, Solanum viarum*
Tomato yellow leaf curl (TYLCV)	Cuba, Dominican Republic, Jamaica, U.S. (Florida)	Reduced leaf size, chlorotic margins, chlorotic mottling, flower abscission, stunting	Host range includes species in 10 families
Tomato yellow mosaic (TYMV)	Venezuela	Yellow mosaic, leaf curling, stunting	*D.stramonium*, 2 *Lycopersicon* spp., *N.physaloides, N..tabacum, Petunia hybrida,*
Tomato yellow mottle (ToYMoV)	Costa Rica	Chlorotic mottle, leaf curling and distortion, stunting	Not reported
Tomato yellow vein streak virus (ToYVSV)	Brazil	Yellow mosaic, wavy leaves	*Solanum tuberosum*

Table A-2. Geographical distribution and economic importance of TYLCV (modified from Pico et al., 1996).

Country	Growing Season	Incidence	Yield Losses
Israel	Summer, autumn and early winter	93-100%	Most significant factor reducing productivity in summer and autumn
Lebanon	September December	28-50% 85-90%	Summer and autumn production of tomato abandoned along coastal
Egypt	Autumn	100%	Most serious disease of tomatoes 80-99%.
Saudi Arabia	August-September plantings December-February plantings	58-100%	Epidemic proportions in summer and early autumn 30-80%.
Cyprus	Summer and early autumn plantings. Winter and early	20-100%	Epidemic proportions. Most limiting factor in tomato production 50-82%.
Tunisia	August plantings September plantings	100% 20%	Affecting tomato production in field and protected crops.
Italy	Greenhouse production, autumn and winter	80%	Severe economic losses in fields and in greenhouses
Spain	Autumn and winter	70%	Important economic losses in fields and in greenhouses.

APPENDIX B
PEDIGREE OF TOMATO LINES USED IN EXPERIMENTS

Table B-1. Source and pedigree of breeding lines used in experiments.

Designation	Seed Source	Pedigree	Resistance Source
960729-16	VIR F6	<7546x{7060x[7420x(7314xLA1932)]-1}-5-11=3>-1-9-9	LA 1932
97E153F	962008x744	Fla. 7324 x 960744	LA 1932
974477	970758-11	7324x[7324x(7309xLA1938)=2]}-1)=5-2-1-14]-BK	LA1938
		-IIID20-11[7324 X (7309Cx{[7324x(7309xLA1938)=6]	
934670	930580-12	[7412x(7309xLA1938)]-4-4-12	LA 1938
960719	954587-12	<7546x{7060x[7420x(7314xLA1932)]-1}-5-11=3>-4-1-3	LA 1932
960724	954589-12	<7546x{7060x[7420x(7314xLA1932)]-1}-5-11=3>-4-1-8	LA 1932
960744	954596-4	<7482Bx{7060x[7420x(7314xLA1932)]-1}-5-11=3>-BK-	LA 1932
97E406S	96E103S	7324X<7482B X{7060X[7420X(7314XLA1932)]-1}	LA 1932
		-5-11=3>-BK-27-7-4=BK)BK	
940699-14	934672-10	7171x[7412x(7309xLA1938)]-4-4-12-5-10	LA 1938
97E121F	P1683	1358 BC2S2 (CROSSED IN ISRAEL)	LA 2779
TY-52	p1606	LA 1969 BC3S3 FROM ZAMIR (*Ty-1*)	LA 1969
TY-50	p1607	LA 1969 BC3S3 FROM ZAMIR (*Ty-1*+)	LA1969
971358-4	p1680	{7409 x (C28 x LA 2779)]-3-1-24-9-9-10 x ISRAELI LINE)]-11-4	LA 2779
97E124F	P1632	(FIONA x <TYKING x{TYKING x [TROP1VA3 x (MOMOR VERTE x LA 1969)]}>) -94-3-6-13-9	LA 1969 /TyKing

APPENDIX C
INHERITANCE ANALYSIS OF FALL 1997 AND SPRING 1998

Table C-1. Adequacy of the additive/dominance model Fall 1997.

Generation	Observed mean	Expected mean	Squared deviation	Chi-square Value
P1	0.61	0.39	0.048	2.99
BC1	1.36	1.44	0.006	1.61
F1	2.50	2.49	0.000	0.04
F2	2.23	2.34	0.001	0.56
BC2	3.33	3.24	0.001	2.94
P2	3.99	3.99	0.000	0.02
-	-	-	-	Total=8.19

Table C-2. Adequacy of the additive/dominance model Spring 1998.

Generation	Observed mean	Expected mean	Squared deviation	Chi-square value
P1	0.83	0.79	0.002	0.24
BC1	1.51	1.55	0.002	0.55
F1	2.49	2.31	0.031	4.53
F2	2.36	2.35	0.000	0.05
BC2	3.00	3.15	0.023	6.58
P2	4.00	3.99	0.000	0.10
-	-	-	-	Total=12.0

Table C-3. Estimates of component of variation, heritability and effective factor number for 719 (LA 1932) a Fla. 7324 cross for combined data from Fall 1997 and Spring 1998.

Variable	Fall 1997	Spring 1998
Additive variance	-1.80	-1.60
Dominance variance	0.30	-0.08
Environmental variance	0.25	0.20
Broad-sense heritability	0.94	0.90
Narrow-sense heritability	1.20	0.63
Effective factor number	1.78	2.29

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BIOGRAPHICAL SKETCH

Phillip David Griffiths was born on 1 July, 1969, to Owen and Pauline Griffiths in Abergavenny, Wales. He received his bachelor of science degree in genetics from the University of Nottingham in 1990 before moving to the University of Wales, where he received a master of science degree in plant breeding in 1993. The topic of the thesis study was "genotypic and environmental effects on endopolyploidy in the epidermal tissues of *Lolium perenne* and *Lolium multiflorum*" and it was supervised by Professor R.N. Jones. In 1994 he moved to Florida to work in a tomato breeding program with Dr. J.W. Scott at the Gulf Coast Research and Education Center, where the research inspired him to pursue a Ph.D. in plant breeding and genetics at the University of Florida.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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